N437.S	3	6	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques, Subdoligranulum variabile	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques, Subdoligranulum variabile
N457.S	5	6	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_522 or clade_522i)	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N545	5	6	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396, (clade_444 or clade_444i), clade_538	Coprococcus comes, Dorea longicatena, Eubacterium hallii, Eubacterium siraeum, Roseburia intestinalis, Roseburia inulinivorans	Coprococcus comes, Dorea longicatena, Eubacterium hallii, Eubacterium siraeum, Roseburia intestinalis, Roseburia inulinivorans
N386.S	5	6	83.3	100	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i), clade_500	Alistipes putredinis, Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques	Alistipes putredinis, Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques
N402.S	5	6	83.3	100	(clade_262 or clade_262i), clade_286, (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Parabacteroides merdae, Ruminococcus torques	Coprococcus comes, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Parabacteroides merdae, Ruminococcus torques
N405.S	4	6	83.3	100	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_445, (clade_478 or clade_478i)	Desulfovibrio desulfuricans, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques, Subdoligranulum variabile	Desulfovibrio desulfuricans, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques, Subdoligranulum variabile

N415.S	5	6	83.3	100	clade_170, (clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Bacteroides caccae, Coprococcus comes, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Bacteroides caccae, Coprococcus comes, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N421.S	6	6	83.3	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i), clade_500	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii, Roseburia inulinivorans, Subdoligranulum variabile	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii, Roseburia inulinivorans, Subdoligranulum variabile
N422.S	5	6	83.3	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), clade_494, clade_500	Alistipes putredinis, Dorea longicatena, Eubacterium rectale, Pseudoflavonifractor capillosus, Roseburia intestinalis, Ruminococcus torques	Alistipes putredinis, Dorea longicatena, Eubacterium rectale, Pseudoflavonifractor capillosus, Roseburia intestinalis, Ruminococcus torques
N423.S	5	6	83.3	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_500, clade_538	Alistipes putredinis, Eubacterium rectale, Eubacterium siraeum, Faecalibacterium prausnitzii, Ruminococcus torques, Subdoligranulum variabile	Alistipes putredinis, Eubacterium rectale, Eubacterium siraeum, Faecalibacterium prausnitzii, Ruminococcus torques, Subdoligranulum variabile
N458.S	6	6	83.3	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_38 or clade_38e or clade_38i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_522 or clade_522i)	Bacteroides ovatus, Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Bacteroides ovatus, Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N459.S	5	6	83.3	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_38 or clade_38e or clade_38i), (clade_444 or clade_444i), (clade_478	Bacteroides ovatus, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques	Bacteroides ovatus, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques

					or clade_478i)		
N493.S	6	6	100	83.3	(clade_262 or clade_262i), (clade_354 or clade_354e), clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494	Clostridium bartlettii, Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N416.S	6	6	83.3	83.3	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), clade_494, clade_500, (clade_516 or clade_516c or clade_516g or clade_516h)	Alistipes putredinis, Anaerotruncus colihominis, Eubacterium hallii, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques	Alistipes putredinis, Eubacterium hallii, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N439.S	6	6	83.3	83.3	(clade_262 or clade_262i), clade_293, (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494	Bifidobacterium bifidum, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N447.S	5	6	83.3	83.3	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494, clade_506	Dialister invisus, Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques, Subdoligranulum variabile	Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques, Subdoligranulum variabile
N490.S	5	6	83.3	83.3	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_98 or clade_98i)	Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques, Streptococcus australis	Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N526	5	6	83.3	83.3	(clade_262 or clade_262i), clade_271, (clade_360 or clade_360c or clade_360g or clade_360h or	Coprococcus comes, Dorea longicatena, Eubacterium hallii, Roseburia intestinalis, Roseburia inulinivorans, Rothia	Coprococcus comes, Dorea longicatena, Eubacterium hallii, Roseburia intestinalis, Roseburia inulinivorans

					clade_360i), clade_396, (clade_444 or clade_444i)	mucilaginosa	
N429.S	4	5	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i), clade_519, (clade_522 or clade_522i)	Eubacterium eligens, Eubacterium ventriosum, Faecalibacterium prausnitzii, Roseburia inulinivorans, Subdoligranulum variabile	Eubacterium eligens, Eubacterium ventriosum, Faecalibacterium prausnitzii, Roseburia inulinivorans, Subdoligranulum variabile
N433.S	4	5	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Coprococcus comes, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N448.S	4	5	100	100	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Eubacterium hallii, Faecalibacterium prausnitzii, Roseburia inulinivorans, Subdoligranulum variabile	Coprococcus comes, Eubacterium hallii, Faecalibacterium prausnitzii, Roseburia inulinivorans, Subdoligranulum variabile
N488.S	4	5	100	100	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques	Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N508.S	4	5	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N509.S	5	5	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_519	Dorea longicatena, Eubacterium rectale, Eubacterium ventriosum, Faecalibacterium prausnitzii, Ruminococcus torques	Dorea longicatena, Eubacterium rectale, Eubacterium ventriosum, Faecalibacterium prausnitzii, Ruminococcus torques

N510.S	5	5	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_522 or clade_522i)	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N511.S	3	5	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N408.S	5	5	80	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_494, clade_500, (clade_522 or clade_522i)	Alistipes putredinis, Eubacterium eligens, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Alistipes putredinis, Eubacterium eligens, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N446.S	5	5	80	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396, (clade_444 or clade_444i), clade_500	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii, Roseburia inulinivorans	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii, Roseburia inulinivorans
N451.S	4	5	80	100	(clade_262 or clade_262i), clade_396, (clade_478 or clade_478i), clade_500	Alistipes putredinis, Coprococcus comes, Eubacterium hallii, Faecalibacterium prausnitzii, Ruminococcus torques	Alistipes putredinis, Coprococcus comes, Eubacterium hallii, Faecalibacterium prausnitzii, Ruminococcus torques
N474.S	5	5	80	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494, (clade_566 or clade_566f)	Eubacterium rectale, Faecalibacterium prausnitzii, Gordonibacter pamelaeae, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Gordonibacter pamelaeae, Pseudoflavonifractor capillosus, Ruminococcus torques
N520.S	5	5	80	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_494, clade_537, (clade_566 or clade_566f)	Clostridium leptum, Faecalibacterium prausnitzii, Gordonibacter pamelaeae, Pseudoflavonifractor capillosus, Ruminococcus torques	Clostridium leptum, Faecalibacterium prausnitzii, Gordonibacter pamelaeae, Pseudoflavonifractor capillosus, Ruminococcus torques

N521.S	5	5	80	100	(clade_262 or clade_262i), clade_393, (clade_444 or clade_444i), clade_445, (clade_478 or clade_478i)	Coprococcus catus, Desulfovibrio desulfuricans, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques	Coprococcus catus, Desulfovibrio desulfuricans, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques
N535.S	4	5	80	100	clade_358, clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium hallii, Faecalibacterium prausnitzii, Roseburia inulinivorans, Subdoligranulum variabile, Veillonella atypica	Eubacterium hallii, Faecalibacterium prausnitzii, Roseburia inulinivorans, Subdoligranulum variabile, Veillonella atypica
N516.S	5	5	60	100	clade_393, (clade_444 or clade_444i), clade_445, clade_521, (clade_522 or clade_522i)	Bilophila wadsworthia, Coprococcus catus, Desulfovibrio desulfuricans, Eubacterium eligens, Eubacterium rectale	Bilophila wadsworthia, Coprococcus catus, Desulfovibrio desulfuricans, Eubacterium eligens, Eubacterium rectale
N463.S	5	5	100	80	clade_246, (clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_576	Clostridiales sp. SS3/4, Clostridium lactatifermentans, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques	Clostridium lactatifermentans, Faecalibacterium prausnitzii, Roseburia inulinivorans, Ruminococcus torques
N518.S	5	5	100	80	(clade_262 or clade_262i), (clade_354 or clade_354e), clade_396, (clade_444 or clade_444i), clade_494	Clostridium bartlettii, Eubacterium hallii, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium hallii, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N586	3	5	100	80	clade_368, (clade_444 or clade_444i), (clade_478 or clade_478i)	Blautia hydrogenotrophica, Eubacterium rectale, Roseburia intestinalis, Roseburia inulinivorans, Subdoligranulum variabile	Eubacterium rectale, Roseburia intestinalis, Roseburia inulinivorans, Subdoligranulum variabile
N450.S	5	5	80	80	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_494, clade_500, (clade_516 or clade_516c or clade_516g or clade_516h)	Alistipes putredinis, Anaerotruncus colihominis, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Alistipes putredinis, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques

N465.S	4	5	80	80	clade_168, (clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Prevotella copri, Roseburia intestinalis, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N519.S	5	5	80	80	(clade_262 or clade_262i), clade_401, (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494	Eubacterium rectale, Faecalibacterium prausnitzii, Lactococcus lactis, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N537.S	5	5	80	80	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_444 or clade_444i), clade_445, clade_494	Clostridium scindens, Desulfovibrio desulfuricans, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques	Desulfovibrio desulfuricans, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N419.S	5	5	60	80	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_494, clade_500, (clade_98 or clade_98i)	Alistipes putredinis, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques, Streptococcus salivarius	Alistipes putredinis, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N468.S	5	5	60	80	(clade_262 or clade_262i), clade_293, (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396, clade_500	Alistipes putredinis, Bifidobacterium bifidum, Coprococcus comes, Dorea longicatena, Eubacterium hallii	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii
N477.S	5	5	60	80	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_494, clade_500, (clade_98 or clade_98i)	Alistipes putredinis, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques, Streptococcus thermophilus	Alistipes putredinis, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N514.S	5	5	60	60	clade_393, (clade_420 or clade_420f), (clade_444 or clade_444i), (clade_522 or clade_522i), clade_558	Clostridiales bacterium oral clone P4PA, Coprococcus catus, Eubacterium eligens, Eubacterium rectale, Tannerella sp. 6_1_58FAA_CT1	Coprococcus catus, Eubacterium eligens, Eubacterium rectale

N382.S	5	5	100	40	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_537, (clade_553 or clade_553i)	Clostridium hylemonae, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Ruminococcus bromii	Coprococcus comes, Ruminococcus bromii
N460.S	3	4	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N462.S	3	4	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_478 or clade_478i)	Coprococcus comes, Dorea longicatena, Faecalibacterium prausnitzii, Subdoligranulum variabile	Coprococcus comes, Dorea longicatena, Faecalibacterium prausnitzii, Subdoligranulum variabile
N512.S	3	4	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i)	Coprococcus comes, Dorea longicatena, Eubacterium rectale, Ruminococcus torques	Coprococcus comes, Dorea longicatena, Eubacterium rectale, Ruminococcus torques
N517.S	4	4	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N523.S	3	4	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus torques
N547.S	3	4	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques, Subdoligranulum variabile	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques, Subdoligranulum variabile

N548.S	4	4	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494	Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N577.S	3	4	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_537	Clostridium leptum, Coprococcus comes, Dorea longicatena, Ruminococcus torques	Clostridium leptum, Coprococcus comes, Dorea longicatena, Ruminococcus torques
N581.S	4	4	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_494, clade_538	Eubacterium siraeum, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium siraeum, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N585.S	2	4	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Roseburia inulinivorans	Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Roseburia inulinivorans
N616.S	4	4	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396, (clade_478 or clade_478i)	Coprococcus comes, Dorea longicatena, Eubacterium hallii, Faecalibacterium prausnitzii	Coprococcus comes, Dorea longicatena, Eubacterium hallii, Faecalibacterium prausnitzii
N466.S	4	4	75	100	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_494, clade_500	Alistipes putredinis, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques	Alistipes putredinis, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N469.S	4	4	75	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396, clade_500	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii	Alistipes putredinis, Coprococcus comes, Dorea longicatena, Eubacterium hallii
N480.S	4	4	75	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_500, clade_537	Alistipes putredinis, Faecalibacterium prausnitzii, Ruminococcus bromii, Ruminococcus torques	Alistipes putredinis, Faecalibacterium prausnitzii, Ruminococcus bromii, Ruminococcus torques
N482.S	4	4	75	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_500, (clade_522 or clade_522i)	Alistipes putredinis, Eubacterium eligens, Faecalibacterium prausnitzii, Ruminococcus torques	Alistipes putredinis, Eubacterium eligens, Faecalibacterium prausnitzii, Ruminococcus torques

N484.S	4	4	75	100	(clade_262 or clade_262i), clade_396, (clade_478 or clade_478i), clade_500	Alistipes putredinis, Eubacterium hallii, Faecalibacterium prausnitzii, Ruminococcus torques	Alistipes putredinis, Eubacterium hallii, Faecalibacterium prausnitzii, Ruminococcus torques
N515.S	4	4	75	100	clade_393, (clade_444 or clade_444i), clade_521, (clade_522 or clade_522i)	Bilophila wadsworthia, Coprococcus catus, Eubacterium eligens, Eubacterium rectale	Bilophila wadsworthia, Coprococcus catus, Eubacterium eligens, Eubacterium rectale
N533.S	4	4	75	100	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_445, clade_494	Desulfovibrio desulfuricans, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques	Desulfovibrio desulfuricans, Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N709	4	4	75	100	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_566 or clade_566f)	Coprococcus comes, Eubacterium hallii, Gordonibacter pamelaeae, Roseburia inulinivorans	Coprococcus comes, Eubacterium hallii, Gordonibacter pamelaeae, Roseburia inulinivorans
N730	4	4	75	100	(clade_262 or clade_262i), clade_286, clade_396, (clade_444 or clade_444i)	Coprococcus comes, Eubacterium hallii, Parabacteroides merdae, Roseburia inulinivorans	Coprococcus comes, Eubacterium hallii, Parabacteroides merdae, Roseburia inulinivorans
N478.S	4	4	50	100	clade_170, (clade_262 or clade_262i), (clade_478 or clade_478i), clade_500	Alistipes putredinis, Bacteroides caccae, Faecalibacterium prausnitzii, Ruminococcus torques	Alistipes putredinis, Bacteroides caccae, Faecalibacterium prausnitzii, Ruminococcus torques
N572.S	4	4	50	100	clade_286, (clade_444 or clade_444i), clade_445, (clade_478 or clade_478i)	Desulfovibrio desulfuricans, Eubacterium rectale, Faecalibacterium prausnitzii, Parabacteroides merdae	Desulfovibrio desulfuricans, Eubacterium rectale, Faecalibacterium prausnitzii, Parabacteroides merdae
N400.S	4	4	100	75	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i)	Clostridium symbiosum, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii
N543.S	4	4	100	75	(clade_262 or clade_262i), (clade_354 or clade_354e), (clade_478 or clade_478i), clade_494	Clostridium bartlettii, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques

N582.S	4	4	100	75	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i)	Clostridium symbiosum, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N621.S	4	4	100	75	clade_396, (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i)	Anaerostipes caccae, Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii	Eubacterium hallii, Eubacterium rectale, Faecalibacterium prausnitzii
N689	4	4	100	75	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_516 or clade_516c or clade_516g or clade_516h)	Clostridium methylpentosum, Coprococcus comes, Eubacterium hallii, Roseburia inulinivorans	Coprococcus comes, Eubacterium hallii, Roseburia inulinivorans
N769	4	4	100	75	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_478 or clade_478i), clade_494	Clostridium scindens, Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N481.S	4	4	75	75	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_500, (clade_553 or clade_553i)	Alistipes putredinis, Collinsella aerofaciens, Faecalibacterium prausnitzii, Ruminococcus torques	Alistipes putredinis, Faecalibacterium prausnitzii, Ruminococcus torques
N525.S	4	4	75	75	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_445, clade_494	Anaerostipes caccae, Desulfovibrio desulfuricans, Pseudoflavonifractor capillosus, Ruminococcus torques	Desulfovibrio desulfuricans, Pseudoflavonifractor capillosus, Ruminococcus torques
N528.S	4	4	75	75	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_445, clade_494	Clostridium symbiosum, Desulfovibrio desulfuricans, Pseudoflavonifractor capillosus, Ruminococcus torques	Desulfovibrio desulfuricans, Pseudoflavonifractor capillosus, Ruminococcus torques

N534.S	4	4	75	75	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_65 or clade_65e)	Bacteroides fragilis, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N574.S	4	4	75	75	clade_396, (clade_478 or clade_478i), clade_537, (clade_98 or clade_98i)	Clostridium leptum, Eubacterium hallii, Faecalibacterium prausnitzii, Streptococcus thermophilus	Clostridium leptum, Eubacterium hallii, Faecalibacterium prausnitzii
N580.S	4	4	75	75	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i), (clade_98 or clade_98i)	Eubacterium hallii, Eubacterium rectale, Ruminococcus torques, Streptococcus australis	Eubacterium hallii, Eubacterium rectale, Ruminococcus torques
N590.S	3	4	75	75	clade_401, (clade_478 or clade_478i), clade_485	Faecalibacterium prausnitzii, Holdemania filiformis, Lactococcus lactis, Subdoligranulum variabile	Faecalibacterium prausnitzii, Holdemania filiformis, Subdoligranulum variabile
N591.S	4	4	75	75	(clade_262 or clade_262i), clade_401, (clade_444 or clade_444i), clade_494	Eubacterium rectale, Lactococcus lactis, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N597.S	3	4	75	75	(clade_478 or clade_478i), (clade_522 or clade_522i), (clade_98 or clade_98i)	Eubacterium eligens, Faecalibacterium prausnitzii, Streptococcus australis, Subdoligranulum variabile	Eubacterium eligens, Faecalibacterium prausnitzii, Subdoligranulum variabile
N664	4	4	75	75	(clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e), (clade_516 or clade_516c or clade_516g or clade_516h), clade_519	Bacteroides dorei, Clostridium methylpentosum, Dorea longicatena, Eubacterium ventriosum	Bacteroides dorei, Dorea longicatena, Eubacterium ventriosum
N693	4	4	75	75	(clade_262 or clade_262i), clade_396, clade_401, (clade_444 or clade_444i)	Coprococcus comes, Eubacterium hallii, Lactococcus lactis, Roseburia inulinivorans	Coprococcus comes, Eubacterium hallii, Roseburia inulinivorans
N530.S	4	4	50	75	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_500, (clade_98 or clade_98i)	Alistipes putredinis, Eubacterium rectale, Ruminococcus torques, Streptococcus australis	Alistipes putredinis, Eubacterium rectale, Ruminococcus torques

N687	4	4	100	50	clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i), clade_543	Bacteroides pectinophilus, Coprococcus eutactus, Faecalibacterium prausnitzii, Roseburia intestinalis	Faecalibacterium prausnitzii, Roseburia intestinalis
N470.S	2	3	100	100	(clade_478 or clade_478i), clade_485	Faecalibacterium prausnitzii, Holdemania filiformis, Subdoligranulum variabile	Faecalibacterium prausnitzii, Holdemania filiformis, Subdoligranulum variabile
N529.S	3	3	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_494	Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques	Faecalibacterium prausnitzii, Pseudoflavonifractor capillosus, Ruminococcus torques
N539.S	2	3	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i)	Eubacterium rectale, Roseburia intestinalis, Ruminococcus torques	Eubacterium rectale, Roseburia intestinalis, Ruminococcus torques
N546.S	3	3	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N570.S	3	3	100	100	(clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii
N579.S	3	3	100	100	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i)	Eubacterium hallii, Eubacterium rectale, Ruminococcus torques	Eubacterium hallii, Eubacterium rectale, Ruminococcus torques
N602.S	3	3	100	100	clade_396, (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium hallii, Roseburia inulinivorans, Subdoligranulum variabile	Eubacterium hallii, Roseburia inulinivorans, Subdoligranulum variabile
N614.S	3	3	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_537	Clostridium leptum, Faecalibacterium prausnitzii, Ruminococcus torques	Clostridium leptum, Faecalibacterium prausnitzii, Ruminococcus torques
N648.S	3	3	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_478 or clade_478i)	Dorea longicatena, Faecalibacterium prausnitzii, Ruminococcus torques	Dorea longicatena, Faecalibacterium prausnitzii, Ruminococcus torques
N652.S	3	3	100	100	clade_393, (clade_444 or clade_444i), (clade_522 or clade_522i)	Coprococcus catus, Eubacterium eligens, Eubacterium rectale	Coprococcus catus, Eubacterium eligens, Eubacterium rectale

N655.S	2	3	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Subdoligranulum variabile	Eubacterium rectale, Faecalibacterium prausnitzii, Subdoligranulum variabile
N672.S	3	3	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_494	Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques	Eubacterium rectale, Pseudoflavonifractor capillosus, Ruminococcus torques
N681.S	2	3	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i)	Coprococcus comes, Faecalibacterium prausnitzii, Ruminococcus torques	Coprococcus comes, Faecalibacterium prausnitzii, Ruminococcus torques
N690.S	3	3	100	100	(clade_262 or clade_262i), clade_396, (clade_444 or clade_444i)	Coprococcus comes, Eubacterium hallii, Roseburia inulinivorans	Coprococcus comes, Eubacterium hallii, Roseburia inulinivorans
N692.S	3	3	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_396	Coprococcus comes, Dorea longicatena, Eubacterium hallii	Coprococcus comes, Dorea longicatena, Eubacterium hallii
N698.S	2	3	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Roseburia intestinalis, Subdoligranulum variabile	Eubacterium rectale, Roseburia intestinalis, Subdoligranulum variabile
N737.S	3	3	100	100	(clade_262 or clade_262i), clade_393, (clade_444 or clade_444i)	Coprococcus catus, Eubacterium rectale, Ruminococcus torques	Coprococcus catus, Eubacterium rectale, Ruminococcus torques
N738.S	3	3	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_485	Faecalibacterium prausnitzii, Holdemania filiformis, Ruminococcus torques	Faecalibacterium prausnitzii, Holdemania filiformis, Ruminococcus torques
N785	3	3	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_519	Coprococcus comes, Dorea longicatena, Eubacterium ventriosum	Coprococcus comes, Dorea longicatena, Eubacterium ventriosum
N841	3	3	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_537	Faecalibacterium prausnitzii, Ruminococcus bromii, Ruminococcus torques	Faecalibacterium prausnitzii, Ruminococcus bromii, Ruminococcus torques
N878	2	3	100	100	(clade_444 or clade_444i), (clade_522 or clade_522i)	Eubacterium eligens, Roseburia intestinalis, Roseburia inulinivorans	Eubacterium eligens, Roseburia intestinalis, Roseburia inulinivorans
N880	2	3	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i)	Coprococcus comes, Eubacterium rectale, Roseburia inulinivorans	Coprococcus comes, Eubacterium rectale, Roseburia inulinivorans
N881	2	3	100	100	clade_396, (clade_444 or clade_444i)	Eubacterium hallii, Roseburia intestinalis, Roseburia inulinivorans	Eubacterium hallii, Roseburia intestinalis, Roseburia inulinivorans

N987	3	3	100	100	(clade_262 or clade_262i), clade_396, clade_537	Coprococcus comes, Eubacterium hallii, Ruminococcus bromii	Coprococcus comes, Eubacterium hallii, Ruminococcus bromii
N988	3	3	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i), clade_537	Eubacterium rectale, Ruminococcus bromii, Subdoligranulum variabile	Eubacterium rectale, Ruminococcus bromii, Subdoligranulum variabile
N996	3	3	100	100	clade_393, clade_396, (clade_444 or clade_444i)	Coprococcus catus, Eubacterium hallii, Roseburia inulinivorans	Coprococcus catus, Eubacterium hallii, Roseburia inulinivorans
N1061	3	3	100	100	(clade_262 or clade_262i), clade_396, (clade_478 or clade_478i)	Eubacterium hallii, Faecalibacterium prausnitzii, Ruminococcus torques	Eubacterium hallii, Faecalibacterium prausnitzii, Ruminococcus torques
N479.S	3	3	66.7	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_500	Alistipes putredinis, Faecalibacterium prausnitzii, Ruminococcus torques	Alistipes putredinis, Faecalibacterium prausnitzii, Ruminococcus torques
N538.S	3	3	66.7	100	(clade_262 or clade_262i), clade_445, clade_494	Desulfovibrio desulfuricans, Pseudoflavonifractor capillosus, Ruminococcus torques	Desulfovibrio desulfuricans, Pseudoflavonifractor capillosus, Ruminococcus torques
N542.S	3	3	66.7	100	(clade_262 or clade_262i), (clade_444 or clade_444i), clade_500	Alistipes putredinis, Eubacterium rectale, Ruminococcus torques	Alistipes putredinis, Eubacterium rectale, Ruminococcus torques
N578.S	3	3	66.7	100	(clade_262 or clade_262i), clade_445, (clade_478 or clade_478i)	Desulfovibrio desulfuricans, Faecalibacterium prausnitzii, Ruminococcus torques	Desulfovibrio desulfuricans, Faecalibacterium prausnitzii, Ruminococcus torques
N609.S	3	3	66.7	100	(clade_262 or clade_262i), clade_286, (clade_444 or clade_444i)	Eubacterium rectale, Parabacteroides merdae, Ruminococcus torques	Eubacterium rectale, Parabacteroides merdae, Ruminococcus torques
N611.S	3	3	66.7	100	(clade_262 or clade_262i), (clade_378 or clade_378e), (clade_478 or clade_478i)	Bacteroides dorei, Faecalibacterium prausnitzii, Ruminococcus torques	Bacteroides dorei, Faecalibacterium prausnitzii, Ruminococcus torques
N617.S	3	3	66.7	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_500	Alistipes putredinis, Dorea longicatena, Ruminococcus torques	Alistipes putredinis, Dorea longicatena, Ruminococcus torques
N666.S	3	3	66.7	100	(clade_444 or clade_444i), clade_521, (clade_522 or clade_522i)	Bilophila wadsworthia, Eubacterium eligens, Eubacterium rectale	Bilophila wadsworthia, Eubacterium eligens, Eubacterium rectale
N675.S	3	3	66.7	100	(clade_262 or clade_262i), (clade_38 or clade_38e or clade_38i), (clade_478 or clade_478i)	Bacteroides ovatus, Faecalibacterium prausnitzii, Ruminococcus torques	Bacteroides ovatus, Faecalibacterium prausnitzii, Ruminococcus torques

N682.S	3	3	66.7	100	clade_170, (clade_444 or clade_444i), (clade_478 or clade_478i)	Bacteroides caccae, Eubacterium rectale, Faecalibacterium prausnitzii	Bacteroides caccae, Eubacterium rectale, Faecalibacterium prausnitzii
N844	3	3	66.7	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), clade_521	Bilophila wadsworthia, Coprococcus comes, Dorea longicatena	Bilophila wadsworthia, Coprococcus comes, Dorea longicatena
N845	3	3	66.7	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e)	Bacteroides dorei, Coprococcus comes, Dorea longicatena	Bacteroides dorei, Coprococcus comes, Dorea longicatena
N846	3	3	66.7	100	clade_170, (clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h clade_360i)	Bacteroides caccae, Coprococcus comes, Dorea longicatena	Bacteroides caccae, Coprococcus comes, Dorea longicatena
N852	3	3	66.7	100	(clade_172 or clade_172i), (clade_262 or clade_262i), (clade_478 or clade_478i)	Bifidobacterium longum, Faecalibacterium prausnitzii, Ruminococcus torques	Bifidobacterium longum, Faecalibacterium prausnitzii, Ruminococcus torques
N876	2	3	66.7	100	clade_170, (clade_444 or clade_444i)	Bacteroides caccae, Roseburia intestinalis, Roseburia inulinivorans	Bacteroides caccae, Roseburia intestinalis, Roseburia inulinivorans
N982	3	3	66.7	100	(clade_172 or clade_172i), (clade_262 or clade_262i), clade_396	Bifidobacterium longum, Coprococcus comes, Eubacterium hallii	Bifidobacterium longum, Coprococcus comes, Eubacterium hallii
N1008	3	3	66.7	100	(clade_262 or clade_262i), (clade_478 or clade_478i), clade_521	Bilophila wadsworthia, Faecalibacterium prausnitzii, Ruminococcus torques	Bilophila wadsworthia, Faecalibacterium prausnitzii, Ruminococcus torques
N649.S	3	3	33.3	100	(clade_378 or clade_378e), (clade_444 or clade_444i), clade_445	Bacteroides dorei, Desulfovibrio desulfuricans, Eubacterium rectale	Bacteroides dorei, Desulfovibrio desulfuricans, Eubacterium rectale
N657.S	3	3	33.3	100	(clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_478 or clade_478i)	Bacteroides dorei, Bacteroides ovatus, Faecalibacterium prausnitzii	Bacteroides dorei, Bacteroides ovatus, Faecalibacterium prausnitzii
N678.S	3	3	33.3	100	clade_445, (clade_478 or clade_478i), clade_583	Akkermansia muciniphila, Desulfovibrio desulfuricans, Faecalibacterium prausnitzii	Akkermansia muciniphila, Desulfovibrio desulfuricans, Faecalibacterium prausnitzii

N686.S	3	3	33.3	100	(clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), clade_485	Bacteroides dorei, Bacteroides ovatus, Holdemania filiformis	Bacteroides dorei, Bacteroides ovatus, Holdemania filiformis
N710.S	3	3	33.3	100	(clade_478 or clade_478i), clade_500, (clade_566 or clade_566f)	Alistipes putredinis, Gordonibacter pamelaeae, Subdoligranulum variabile	Alistipes putredinis, Gordonibacter pamelaeae, Subdoligranulum variabile
N522.S	3	3	100	66.7	(clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_538	Clostridium symbiosum, Eubacterium siraeum, Pseudoflavonifractor capillosus	Eubacterium siraeum, Pseudoflavonifractor capillosus
N651.S	3	3	100	66.7	(clade_444 or clade_444i), (clade_516 or clade_516c or clade_516g or clade_516h), (clade_522 or clade_522i)	Anaerotruncus colihominis, Eubacterium eligens, Roseburia inulinivorans	Eubacterium eligens, Roseburia inulinivorans
N653.S	3	3	100	66.7	clade_396, (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i)	Anaerostipes caccae, Eubacterium hallii, Eubacterium rectale	Eubacterium hallii, Eubacterium rectale
N654.S	3	3	100	66.7	(clade_444 or clade_444i), (clade_478 or clade_478i), (clade_516 or clade_516c or clade_516g or clade_516h)	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus albus	Eubacterium rectale, Faecalibacterium prausnitzii
N680.S	3	3	100	66.7	(clade_262 or clade_262i), clade_368, (clade_478 or clade_478i)	Blautia hydrogenotrophica, Faecalibacterium prausnitzii, Ruminococcus torques	Faecalibacterium prausnitzii, Ruminococcus torques
N712.S	2	3	100	66.7	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_478 or clade_478i)	Clostridium scindens, Faecalibacterium prausnitzii, Subdoligranulum variabile	Faecalibacterium prausnitzii, Subdoligranulum variabile
N792	3	3	100	66.7	(clade_444 or clade_444i), (clade_478 or clade_478i), (clade_516 or clade_516c or clade_516g or clade_516h)	Clostridium methylpentosum, Eubacterium rectale, Subdoligranulum variabile	Eubacterium rectale, Subdoligranulum variabile
N802	3	3	100	66.7	(clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_485, clade_537	Anaerostipes caccae, Clostridium leptum, Holdemania filiformis	Clostridium leptum, Holdemania filiformis

N804	3	3	100	66.7	clade_485, clade_494, clade_543	Coprococcus eutactus, Holdemania filiformis, Pseudoflavonifractor capillosus	Holdemania filiformis, Pseudoflavonifractor capillosus
N807	3	3	100	66.7	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_478 or clade_478i)	Anaerostipes caccae, Coprococcus comes, Subdoligranulum variabile	Coprococcus comes, Subdoligranulum variabile
N849	3	3	100	66.7	(clade_444 or clade_444i), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_522 or clade_522i)	Clostridium cocleatum, Eubacterium eligens, Eubacterium rectale	Eubacterium eligens, Eubacterium rectale
N858	3	3	100	66.7	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494	Anaerostipes caccae, Pseudoflavonifractor capillosus, Ruminococcus torques	Pseudoflavonifractor capillosus, Ruminococcus torques
N859	3	3	100	66.7	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), clade_494	Clostridium scindens, Pseudoflavonifractor capillosus, Ruminococcus torques	Pseudoflavonifractor capillosus, Ruminococcus torques
N875	2	3	100	66.7	(clade_444 or clade_444i), (clade_516 or clade_516c or clade_516g or clade_516h)	Anaerotruncus colihominis, Roseburia intestinalis, Roseburia inulinivorans	Roseburia intestinalis, Roseburia inulinivorans
N885	3	3	100	66.7	(clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_478 or clade_478i), clade_485	Anaerostipes caccae, Faecalibacterium prausnitzii, Holdemania filiformis	Faecalibacterium prausnitzii, Holdemania filiformis
N942	3	3	100	66.7	(clade_260 or clade_260c or clade_260g or clade_260h), clade_396, (clade_444 or clade_444i)	Clostridium scindens, Eubacterium hallii, Roseburia inulinivorans	Eubacterium hallii, Roseburia inulinivorans
N961	3	3	100	66.7	(clade_354 or clade_354e), (clade_444 or clade_444i), (clade_478 or clade_478i)	Clostridium bartlettii, Eubacterium rectale, Subdoligranulum variabile	Eubacterium rectale, Subdoligranulum variabile

N972	3	3	100	66.7	(clade_444 or clade_444i), (clade_478 or clade_478i), (clade_516 or clade_516c or clade_516g or clade_516h)	Anaerotruncus colihominis, Eubacterium rectale, Subdoligranulum variabile	Eubacterium rectale, Subdoligranulum variabile
N1051	3	3	100	66.7	(clade_262 or clade_262i), (clade_478 or clade_478i), (clade_516 or clade_516c or clade_516g or clade_516h)	Anaerotruncus colihominis, Faecalibacterium prausnitzii, Ruminococcus torques	Faecalibacterium prausnitzii, Ruminococcus torques
N587.S	3	3	66.7	66.7	(clade_478 or clade_478i), clade_500, (clade_516 or clade_516c or clade_516g or clade_516h)	Alistipes putredinis, Clostridium methylpentosum, Subdoligranulum variabile	Alistipes putredinis, Subdoligranulum variabile
N589.S	3	3	66.7	66.7	(clade_262 or clade_262i), clade_293, (clade_478 or clade_478i)	Bifidobacterium bifidum, Faecalibacterium prausnitzii, Ruminococcus torques	Faecalibacterium prausnitzii, Ruminococcus torques
N612.S	3	3	66.7	66.7	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_98 or clade_98i)	Eubacterium rectale, Ruminococcus torques, Streptococcus salivarius	Eubacterium rectale, Ruminococcus torques
N625.S	3	3	66.7	66.7	(clade_444 or clade_444i), (clade_478 or clade_478i), (clade_98 or clade_98i)	Eubacterium rectale, Faecalibacterium prausnitzii, Streptococcus salivarius	Eubacterium rectale, Faecalibacterium prausnitzii
N656.S	3	3	66.7	66.7	clade_170, clade_396, clade_537	Bacteroides caccae, Bacteroides pectinophilus, Clostridium leptum	Bacteroides caccae, Clostridium leptum
N714.S	2	3	66.7	66.7	(clade_478 or clade_478i), (clade_98 or clade_98i)	Faecalibacterium prausnitzii, Streptococcus thermophilus, Subdoligranulum variabile	Faecalibacterium prausnitzii, Subdoligranulum variabile
N779	3	3	66.7	66.7	(clade_444 or clade_444i), (clade_478 or clade_478i), (clade_98 or clade_98i)	Faecalibacterium prausnitzii, Roseburia intestinalis, Streptococcus australis	Faecalibacterium prausnitzii, Roseburia intestinalis
N781	3	3	66.7	66.7	clade_293, clade_393, (clade_444 or clade_444i)	Bifidobacterium bifidum, Coprococcus catus, Eubacterium rectale	Coprococcus catus, Eubacterium rectale
N828	3	3	66.7	66.7	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_98 or clade_98i)	Coprococcus comes, Dorea longicatena, Streptococcus australis	Coprococcus comes, Dorea longicatena

N829	3	3	66.7	66.7	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_65 or clade_65e)	Bacteroides fragilis, Coprococcus comes, Dorea longicatena	Coprococcus comes, Dorea longicatena
N860	3	3	66.7	66.7	(clade_262 or clade_262i), clade_494, (clade_98 or clade_98i)	Pseudoflavonifractor capillosus, Ruminococcus torques, Streptococcus thermophilus	Pseudoflavonifractor capillosus, Ruminococcus torques
N894	3	3	66.7	66.7	clade_293, (clade_444 or clade_444i), (clade_478 or clade_478i)	Bifidobacterium bifidum, Eubacterium rectale, Subdoligranulum variabile	Eubacterium rectale, Subdoligranulum variabile
N925	3	3	66.7	66.7	clade_396, (clade_444 or clade_444i), (clade_98 or clade_98i)	Eubacterium hallii, Roseburia inulinivorans, Streptococcus australis	Eubacterium hallii, Roseburia inulinivorans
N927	3	3	66.7	66.7	clade_396, (clade_444 or clade_444i), clade_506	Dialister invisus, Eubacterium hallii, Roseburia inulinivorans	Eubacterium hallii, Roseburia inulinivorans
N935	3	3	66.7	66.7	clade_396, (clade_444 or clade_444i), (clade_65 or clade_65e)	Bacteroides fragilis, Eubacterium hallii, Roseburia inulinivorans	Eubacterium hallii, Roseburia inulinivorans
N947	3	3	66.7	66.7	clade_396, (clade_444 or clade_444i), (clade_98 or clade_98i)	Eubacterium hallii, Roseburia inulinivorans, Streptococcus thermophilus	Eubacterium hallii, Roseburia inulinivorans
N983	3	3	66.7	66.7	clade_396, (clade_444 or clade_444i), (clade_98 or clade_98i)	Eubacterium hallii, Roseburia inulinivorans, Streptococcus salivarius	Eubacterium hallii, Roseburia inulinivorans
N1023	3	3	66.7	66.7	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_98 or clade_98i)	Dorea longicatena, Ruminococcus torques, Streptococcus salivarius	Dorea longicatena, Ruminococcus torques
N441.S	3	3	100	33.3	clade_252, (clade_262 or clade_262i), (clade_354 or clade_354e)	Clostridium butyricum, Eubacterium tenue, Ruminococcus torques	Ruminococcus torques
N584.S	3	3	100	33.3	(clade_262 or clade_262i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_553 or clade_553i)	Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes	Coprococcus comes
N794	3	3	100	33.3	(clade_516 or clade_516c or clade_516g or clade_516h), clade_537, clade_543	Anaerotruncus colihominis, Clostridium leptum, Coprococcus eutactus	Clostridium leptum

N788	1	3	100	0	(clade_516 or clade_516c or clade_516g or clade_516h)	Anaerotruncus colihominis, Clostridium methylpentosum, Ruminococcus albus	
N524.S	1	2	100	100	(clade_478 or clade_478i)	Faecalibacterium prausnitzii, Subdoligranulum variabile	Faecalibacterium prausnitzii, Subdoligranulum variabile
N604.S	2	2	100	100	(clade_262 or clade_262i), (clade_478 or clade_478i)	Faecalibacterium prausnitzii, Ruminococcus torques	Faecalibacterium prausnitzii, Ruminococcus torques
N610.S	2	2	100	100	(clade_262 or clade_262i), (clade_444 or clade_444i)	Eubacterium rectale, Ruminococcus torques	Eubacterium rectale, Ruminococcus torques
N623.S	2	2	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii	Eubacterium rectale, Faecalibacterium prausnitzii
N663.S	2	2	100	100	(clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i)	Dorea longicatena, Roseburia inulinivorans	Dorea longicatena, Roseburia inulinivorans
N669.S	2	2	100	100	(clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h clade_360i)	Coprococcus comes, Dorea longicatena	Coprococcus comes, Dorea longicatena
N676.S	2	2	100	100	(clade_262 or clade_262i), clade_494	Pseudoflavonifractor capillosus, Ruminococcus torques	Pseudoflavonifractor capillosus, Ruminococcus torques
N703.S	2	2	100	100	(clade_478 or clade_478i), clade_485	Faecalibacterium prausnitzii, Holdemania filiformis	Faecalibacterium prausnitzii, Holdemania filiformis
N775.S	2	2	100	100	(clade_262 or clade_262i), clade_396	Coprococcus comes, Eubacterium hallii	Coprococcus comes, Eubacterium hallii
N777.S	2	2	100	100	(clade_444 or clade_444i), clade_519	Eubacterium ventriosum, Roseburia inulinivorans	Eubacterium ventriosum, Roseburia inulinivorans
N780.S	2	2	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i)	Faecalibacterium prausnitzii, Roseburia intestinalis	Faecalibacterium prausnitzii, Roseburia intestinalis
N817.S	2	2	100	100	clade_519, clade_537	Clostridium leptum, Eubacterium ventriosum	Clostridium leptum, Eubacterium ventriosum
N827.S	2	2	100	100	(clade_444 or clade_444i), (clade_522 or clade_522i)	Eubacterium eligens, Eubacterium rectale	Eubacterium eligens, Eubacterium rectale
N836.S	2	2	100	100	(clade_478 or clade_478i), clade_494	Pseudoflavonifractor capillosus, Subdoligranulum variabile	Pseudoflavonifractor capillosus, Subdoligranulum variabile

N871.S	1	2	100	100	(clade_444 or clade_444i)	Roseburia intestinalis, Roseburia inulinivorans	Roseburia intestinalis, Roseburia inulinivorans
N874.S	2	2	100	100	clade_396, (clade_444 or clade_444i)	Eubacterium hallii, Roseburia inulinivorans	Eubacterium hallii, Roseburia inulinivorans
N898.S	2	2	100	100	(clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Subdoligranulum variabile	Eubacterium rectale, Subdoligranulum variabile
N907.S	2	2	100	100	(clade_360 or clade_360e or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i)	Dorea longicatena, Roseburia intestinalis	Dorea longicatena, Roseburia intestinalis
N998.S	2	2	100	100	clade_393, (clade_478 or clade_478i)	Coprococcus catus, Faecalibacterium prausnitzii	Coprococcus catus, Faecalibacterium prausnitzii
N1088	2	2	100	100	clade_393, (clade_444 or clade_444i)	Coprococcus catus, Eubacterium rectale	Coprococcus catus, Eubacterium rectale
N1089	2	2	100	100	(clade_478 or clade_478i), clade_576	Clostridium lactatifermentans, Faecalibacterium prausnitzii	Clostridium lactatifermentans, Faecalibacterium prausnitzii
N660.S	2	2	50	100	(clade_172 or clade_172i), clade_485	Bifidobacterium longum, Holdemania filiformis	Bifidobacterium longum, Holdemania filiformis
N665.S	2	2	50	100	clade_286, (clade_444 or clade_444i)	Parabacteroides merdae, Roseburia intestinalis	Parabacteroides merdae, Roseburia intestinalis
N667.S	2	2	50	100	clade_500, (clade_522 or clade_522i)	Alistipes putredinis, Eubacterium eligens	Alistipes putredinis, Eubacterium eligens
N733.S	2	2	50	100	(clade_262 or clade_262i), (clade_38 or clade_38e or clade_38i)	Bacteroides ovatus, Ruminococcus torques	Bacteroides ovatus, Ruminococcus torques
N734.S	2	2	50	100	(clade_262 or clade_262i), clade_521	Bilophila wadsworthia, Ruminococcus torques	Bilophila wadsworthia, Ruminococcus torques
N739.S	2	2	50	100	(clade_478 or clade_478i), clade_500	Alistipes putredinis, Faecalibacterium prausnitzii	Alistipes putredinis, Faecalibacterium prausnitzii
N741.S	2	2	50	100	(clade_262 or clade_262i), clade_500	Alistipes putredinis, Ruminococcus torques	Alistipes putredinis, Ruminococcus torques
N782.S	2	2	50	100	clade_171, (clade_522 or clade_522i)	Bacteroides intestinalis, Eubacterium eligens	Bacteroides intestinalis, Eubacterium eligens
N789.S	2	2	50	100	clade_357, clade_537	Clostridium leptum, Oxalobacter formigenes	Clostridium leptum, Oxalobacter formigenes
N796.S	2	2	50	100	clade_500, clade_537	Alistipes putredinis, Ruminococcus bromii	Alistipes putredinis, Ruminococcus bromii
N798.S	2	2	50	100	(clade_378 or clade_378e), clade_494	Bacteroides dorei, Pseudoflavonifractor capillosus	Bacteroides dorei, Pseudoflavonifractor capillosus
N800.S	2	2	50	100	clade_445, clade_537	Clostridium leptum, Desulfovibrio desulfuricans	Clostridium leptum, Desulfovibrio desulfuricans

N809.S	2	2	50	100	clade_445, (clade_478 or clade_478i)	Desulfovibrio desulfuricans, Faecalibacterium prausnitzii	Desulfovibrio desulfuricans, Faecalibacterium prausnitzii
N816.S	2	2	50	100	(clade_38 or clade_38e or clade_38i), (clade_522 or clade_522i)	Bacteroides ovatus, Eubacterium eligens	Bacteroides ovatus, Eubacterium eligens
N842.S	2	2	50	100	clade_393, clade_500	Alistipes putredinis, Coprococcus catus	Alistipes putredinis, Coprococcus catus
N843.S	2	2	50	100	(clade_262 or clade_262i), clade_500	Alistipes putredinis, Coprococcus comes	Alistipes putredinis, Coprococcus comes
N869.S	2	2	50	100	clade_537, (clade_566 or clade_566f)	Clostridium leptum, Gordonibacter pamelaeae	Clostridium leptum, Gordonibacter pamelaeae
N986.S	2	2	50	100	(clade_172 or clade_172i), (clade_262 or clade_262i)	Bifidobacterium longum, Ruminococcus torques	Bifidobacterium longum, Ruminococcus torques
N995.S	2	2	50	100	clade_500, clade_537	Alistipes putredinis, Clostridium leptum	Alistipes putredinis, Clostridium leptum
N1002.S	2	2	50	100	(clade_478 or clade_478i), clade_500	Alistipes putredinis, Subdoligranulum variabile	Alistipes putredinis, Subdoligranulum variabile
N1004.S	2	2	50	100	(clade_262 or clade_262i), clade_445	Desulfovibrio desulfuricans, Ruminococcus torques	Desulfovibrio desulfuricans, Ruminococcus torques
N1019.S	2	2	50	100	clade_358, (clade_478 or clade_478i)	Faecalibacterium prausnitzii, Veillonella atypica	Faecalibacterium prausnitzii, Veillonella atypica
N1093	2	2	50	100	(clade_522 or clade_522i), clade_583	Akkermansia muciniphila, Eubacterium eligens	Akkermansia muciniphila, Eubacterium eligens
N668.S	2	2	0	100	(clade_378 or clade_378e), clade_500	Alistipes putredinis, Bacteroides dorei	Alistipes putredinis, Bacteroides dorei
N685.S	2	2	0	100	clade_170, clade_286	Bacteroides caccae, Parabacteroides merdae	Bacteroides caccae, Parabacteroides merdae
N835.S	2	2	0	100	clade_500, (clade_566 or clade_566f)	Alistipes putredinis, Gordonibacter pamelaeae	Alistipes putredinis, Gordonibacter pamelaeae
N851.S	2	2	0	100	clade_445, clade_521	Bilophila wadsworthia, Desulfovibrio desulfuricans	Bilophila wadsworthia, Desulfovibrio desulfuricans
N464.S	2	2	100	50	clade_252, (clade_262 or clade_262i)	Clostridium butyricum, Ruminococcus torques	Ruminococcus torques
N695.S	2	2	100	50	clade_396, (clade_516 or clade_516c or clade_516g or clade_516h)	Eubacterium hallii, Ruminococcus albus	Eubacterium hallii
N776.S	2	2	100	50	(clade_516 or clade_516c or clade_516g or clade_516h), clade_538	Eubacterium siraeum, Ruminococcus albus	Eubacterium siraeum
N793.S	2	2	100	50	(clade_516 or clade_516c or clade_516g or	Anaerotruncus colihominis, Clostridium	Clostridium leptum

					clade_516h), clade_537	leptum	
N815.S	2	2	100	50	(clade_522 or clade_522i), (clade_553 or clade_553i)	Collinsella aerofaciens, Eubacterium eligens	Eubacterium eligens
N833.S	2	2	100	50	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i)	Clostridium scindens, Coprococcus comes	Coprococcus comes
N891.S	2	2	100	50	clade_368, clade_485	Blautia hydrogenotrophica, Holdemania filiformis	Holdemania filiformis
N1070.S	2	2	100	50	(clade_262 or clade_262i), (clade_516 or clade_516c or clade_516g or clade_516h)	Anaerotruncus colihominis, Coprococcus comes	Coprococcus comes
N1092	2	2	100	50	clade_393, (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i)	Clostridium cocleatum, Coprococcus catus	Coprococcus catus
N795.S	2	2	50	50	clade_500, (clade_516 or clade_516c or clade_516g or clade_516h)	Alistipes putredinis, Clostridium methylpentosum	Alistipes putredinis
N797.S	2	2	50	50	(clade_478 or clade_478i), (clade_98 or clade_98i)	Faecalibacterium prausnitzii, Streptococcus salivarius	Faecalibacterium prausnitzii
N808.S	2	2	50	50	clade_445, (clade_516 or clade_516c or clade_516g or clade_516h)	Clostridium methylpentosum, Desulfovibrio desulfuricans	Desulfovibrio desulfuricans
N811.S	2	2	50	50	clade_445, (clade_516 or clade_516c or clade_516g or clade_516h)	Desulfovibrio desulfuricans, Ruminococcus albus	Desulfovibrio desulfuricans
N826.S	2	2	50	50	clade_500, clade_543	Alistipes putredinis, Coprococcus eutactus	Alistipes putredinis
N830.S	2	2	50	50	(clade_262 or clade_262i), (clade_98 or clade_98i)	Coprococcus comes, Streptococcus thermophilus	Coprococcus comes
N832.S	2	2	50	50	clade_293, (clade_478 or clade_478i)	Bifidobacterium bifidum, Subdoligranulum variabile	Subdoligranulum variabile
N840.S	2	2	50	50	(clade_444 or clade_444i), (clade_98 or clade_98i)	Eubacterium rectale, Streptococcus salivarius	Eubacterium rectale
N945.S	2	2	50	50	(clade_260 or clade_260c or clade_260g or clade_260h), clade_500	Alistipes putredinis, Clostridium scindens	Alistipes putredinis
N960.S	2	2	50	50	(clade_354 or clade_354e), clade_500	Alistipes putredinis, Clostridium bartlettii	Alistipes putredinis

N968.S	2	2	50	50	(clade_262 or clade_262i), (clade_92 or clade_92e or clade_92i)	Escherichia coli, Ruminococcus torques	Ruminococcus torques
N1091	2	2	50	50	(clade_444 or clade_444i), clade_561	Alistipes indistinctus, Eubacterium rectale	Eubacterium rectale
N805.S	2	2	0	50	clade_293, clade_445	Bifidobacterium bifidum, Desulfovibrio desulfuricans	Desulfovibrio desulfuricans
N822.S	2	2	0	50	clade_401, clade_500	Alistipes putredinis, Lactococcus lactis	Alistipes putredinis
N928.S	2	2	0	50	clade_500, clade_506	Alistipes putredinis, Dialister invisus	Alistipes putredinis
N936.S	2	2	0	50	clade_500, (clade_65 or clade_65e)	Alistipes putredinis, Bacteroides fragilis	Alistipes putredinis
N1078.S	2	2	0	50	clade_445, (clade_98 or clade_98i)	Desulfovibrio desulfuricans, Streptococcus australis	Desulfovibrio desulfuricans
N913.S	2	2	100	0	(clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_516 or clade_516c or clade_516g or clade_516h)	Anaerotruncus colihominis, Clostridium symbiosum	

Table 14b.

Percentage of clinical ethanol-treated spore preparations, or combined engrafted and augmented ecologies of patient's post-treatment with the bacterial composition in which the Network Ecology is observed. Network Ecologies found in doses and patients post treatment comprised 2-15 OTUs. Network Ecology IDs with a ".s" indicates that the network is a subset of the computationally determined networks reported in Table 8 with the same Network Ecology.

Network Ecology ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Cases	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N262.S	15	0	0	10	0	10
N290.S	14	0	0	10	20	10
N271.S	13	0	0	9	0	10
N282.S	13	0	0	9	0	10
N284.S	13	0	0	9	0	10
N302.S	12	0	0	8	20	10
N279.S	12	0	0	9	0	10
N288.S	12	0	0	8	0	10
N310.S	11	0	0	7	20	10
N323.S	11	0	0	7	20	10
N331.S	11	0	0	7	0	10
N332.S	11	0	0	8	0	10
N312.S	10	0	0	7	20	10
N325.S	10	0	0	8	20	10
N338.S	10	0	0	7	20	10
N339.S	10	0	0	7	20	10
N340.S	10	0	0	7	20	10
N341.S	10	0	0	7	20	10
N301.S	10	0	0	7	0	10
N346.S	10	0	0	7	0	10
N381.S	9	0	10	7	70	70
N343.S	9	10	0	7	20	0
N336.S	9	0	0	6	20	10
N353.S	9	0	0	7	20	0
N355.S	9	0	0	6	20	10
N356.S	9	0	0	6	20	10
N361.S	9	0	0	6	20	10
N329.S	9	0	0	6	10	0
N345.S	9	0	0	7	0	10
N344.S	8	10	0	6	20	10

Network Ecologic ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N374.S	8	10	0	6	20	10
N375.S	8	10	0	6	20	10
N352.S	8	0	0	5	20	10
N358.S	8	0	0	5	20	10
N368.S	8	0	0	6	20	10
N369.S	8	0	0	6	20	10
N372.S	8	0	0	6	20	10
N377.S	8	0	0	6	20	0
N380.S	8	0	10	7	10	10
N357.S	8	0	0	6	0	10
N370.S	7	10	0	5	20	10
N389.S	7	10	0	6	20	10
N390.S	7	10	0	6	20	0
N394.S	7	10	0	5	20	10
N396.S	7	10	0	6	20	0
N376.S	7	0	0	5	20	10
N431.S	7	0	10	6	10	10
N434.S	7	0	10	6	10	10
N387.S	7	10	0	6	10	0
N397.S	7	0	0	6	10	0
N440.S	7	0	0	7	10	0
N373.S	7	0	0	6	0	10
N403.S	6	0	50	4	100	80
N432.S	6	0	50	4	100	70
N399.S	6	60	30	6	100	80
N493.S	6	50	30	6	100	70
N436.S	6	20	30	4	100	80
N437.S	6	20	30	3	100	80
N490.S	6	0	0	5	100	40
N414.S	6	40	50	5	70	80
N457.S	6	0	40	5	70	80
N430.S	6	30	40	6	60	50
N402.S	6	50	20	5	60	20
N447.S	6	20	10	5	50	20
N439.S	6	20	0	6	50	0
N415.S	6	20	10	5	30	10
N422.S	6	0	10	5	20	10
N421.S	6	20	0	6	20	10

Network Ecology ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N386.S	6	10	0	5	20	10
N416.S	6	0	0	6	20	10
N458.S	6	0	10	6	10	10
N459.S	6	0	10	5	10	10
N405.S	6	0	0	4	10	0
N526	6	0	0	5	10	0
N423.S	6	0	10	5	0	10
N545	6	0	0	5	0	10
N433.S	5	90	60	4	100	80
N508.S	5	0	50	4	100	80
N511.S	5	0	50	3	100	80
N488.S	5	0	40	4	100	80
N518.S	5	50	30	5	100	70
N448.S	5	30	30	4	100	80
N474.S	5	40	10	5	100	60
N520.S	5	20	10	5	100	50
N509.S	5	70	50	5	80	50
N510.S	5	40	50	5	70	80
N519.S	5	40	30	5	60	30
N429.S	5	0	30	4	60	50
N463.S	5	0	30	5	50	50
N382.S	5	20	0	5	50	0
N514.S	5	20	0	5	50	10
N450.S	5	0	10	5	20	10
N477.S	5	0	10	5	20	10
N535.S	5	0	10	4	20	30
N446.S	5	20	0	5	20	10
N468.S	5	20	0	5	20	0
N451.S	5	10	0	4	20	10
N408.S	5	0	0	5	20	10
N419.S	5	0	0	5	20	10
N465.S	5	0	0	4	20	0
N586	5	0	0	3	20	0
N516.S	5	10	0	5	10	0
N521.S	5	10	0	5	10	0
N537.S	5	10	0	5	10	0
N512.S	4	90	70	3	100	80
N460.S	4	90	60	3	100	80

Network Ecologic ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N517.S	4	90	60	4	100	80
N400.S	4	60	60	4	100	80
N577.S	4	60	60	3	100	70
N582.S	4	50	60	4	100	80
N462.S	4	100	50	3	100	80
N547.S	4	90	50	3	100	80
N548.S	4	70	50	4	100	80
N543.S	4	60	50	4	100	70
N523.S	4	0	50	3	100	80
N616.S	4	90	40	4	100	80
N769	4	40	40	4	100	70
N585.S	4	0	30	2	100	90
N689	4	10	20	4	100	70
N687	4	0	20	4	100	90
N621.S	4	40	10	4	100	90
N574.S	4	60	0	4	100	50
N709	4	30	0	4	100	60
N580.S	4	20	0	4	100	40
N664	4	20	10	4	70	10
N515.S	4	40	0	4	70	0
N597.S	4	0	0	3	70	50
N591.S	4	40	30	4	60	30
N730	4	30	10	4	60	20
N693	4	10	10	4	60	30
N534.S	4	30	40	4	40	40
N590.S	4	30	30	3	30	30
N466.S	4	0	10	4	20	10
N480.S	4	0	10	4	20	10
N469.S	4	20	0	4	20	10
N478.S	4	10	0	4	20	0
N484.S	4	10	0	4	20	10
N482.S	4	0	0	4	20	10
N530.S	4	0	0	4	20	10
N481.S	4	10	0	4	10	0
N533.S	4	10	0	4	10	0
N572.S	4	10	0	4	10	0
N525.S	4	0	0	4	10	0
N528.S	4	0	0	4	10	0

Network Ecologic ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N581.S	4	0	10	4	0	10
N737.S	3	90	70	3	100	80
N672.S	3	70	70	3	100	80
N570.S	3	100	60	3	100	90
N655.S	3	100	60	2	100	90
N546.S	3	90	60	3	100	80
N648.S	3	90	60	3	100	80
N681.S	3	90	60	2	100	80
N961	3	90	60	3	100	80
N579.S	3	80	60	3	100	80
N614.S	3	60	60	3	100	70
N539.S	3	0	60	2	100	80
N698.S	3	0	60	2	100	90
N692.S	3	90	50	3	100	80
N529.S	3	70	50	3	100	80
N859	3	40	50	3	100	70
N1061	3	80	40	3	100	80
N841	3	60	40	3	100	70
N988	3	60	40	3	100	80
N858	3	50	40	3	100	80
N602.S	3	30	40	3	100	90
N792	3	20	40	3	100	70
N1051	3	0	40	3	100	70
N881	3	0	40	2	100	90
N972	3	0	40	3	100	70
N712.S	3	60	30	2	100	80
N807	3	50	30	3	100	80
N653.S	3	40	30	3	100	90
N690.S	3	30	30	3	100	80
N880	3	30	30	2	100	80
N996	3	30	30	3	100	90
N714.S	3	100	20	2	100	50
N987	3	50	20	3	100	70
N942	3	20	20	3	100	80
N875	3	0	20	2	100	70
N860	3	70	10	3	100	40
N1023	3	0	10	3	100	40
N612.S	3	0	10	3	100	40

Network Ecologic ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N625.S	3	0	10	3	100	50
N794	3	0	10	3	100	70
N844	3	100	0	3	100	0
N1008	3	90	0	3	100	0
N947	3	30	0	3	100	50
N654.S	3	20	0	3	100	70
N828	3	20	0	3	100	40
N982	3	20	0	3	100	50
N852	3	10	0	3	100	50
N779	3	0	0	3	100	50
N788	3	0	0	1	100	80
N925	3	0	0	3	100	50
N983	3	0	0	3	100	50
N785	3	80	50	3	80	50
N845	3	80	20	3	80	30
N611.S	3	70	20	3	80	30
N652.S	3	40	60	3	70	90
N878	3	0	40	2	70	90
N849	3	20	20	3	70	90
N651.S	3	0	20	3	70	70
N666.S	3	40	0	3	70	0
N441.S	3	10	0	3	70	50
N609.S	3	50	20	3	60	20
N781	3	50	0	3	50	0
N894	3	50	0	3	50	0
N589.S	3	40	0	3	50	0
N584.S	3	30	0	3	50	10
N927	3	30	0	3	50	20
N738.S	3	40	60	3	40	70
N470.S	3	40	50	2	40	70
N829	3	40	40	3	40	40
N802	3	30	30	3	40	70
N885	3	30	20	3	40	70
N804	3	20	20	3	40	70
N935	3	20	20	3	40	40
N656.S	3	30	10	3	30	10
N682.S	3	30	10	3	30	10
N846	3	30	10	3	30	10

Network Ecologic ID	Number of CUIs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N876	3	0	10	2	30	10
N710.S	3	20	10	3	20	10
N479.S	3	10	10	3	20	10
N542.S	3	10	10	3	20	10
N587.S	3	10	10	3	20	10
N617.S	3	10	10	3	20	10
N680.S	3	20	0	3	20	0
N657.S	3	10	10	3	10	10
N675.S	3	0	10	3	10	10
N538.S	3	10	0	3	10	0
N578.S	3	10	0	3	10	0
N649.S	3	10	0	3	10	0
N678.S	3	10	0	3	10	0
N522.S	3	0	10	3	0	10
N686.S	3	0	10	3	0	10
N1088	2	100	80	2	100	90
N898.S	2	100	80	2	100	90
N610.S	2	90	80	2	100	80
N623.S	2	100	70	2	100	90
N669.S	2	100	70	2	100	80
N998.S	2	100	70	2	100	90
N836.S	2	80	70	2	100	90
N676.S	2	70	70	2	100	80
N524.S	2	100	60	1	100	90
N604.S	2	90	60	2	100	80
N793.S	2	0	60	2	100	80
N775.S	2	90	50	2	100	80
N780.S	2	0	50	2	100	90
N907.S	2	0	50	2	100	90
N913.S	2	0	50	2	100	80
N833.S	2	60	40	2	100	70
N874.S	2	30	40	2	100	90
N1070.S	2	0	40	2	100	70
N871.S	2	0	40	1	100	90
N663.S	2	30	30	2	100	90
N1092	2	80	20	2	100	90
N830.S	2	100	10	2	100	40
N869.S	2	50	10	2	100	50

Network Ecology ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N797.S	2	0	10	2	100	50
N840.S	2	0	10	2	100	50
N734.S	2	90	0	2	100	0
N695.S	2	20	0	2	100	70
N986.S	2	10	0	2	100	50
N817.S	2	60	60	2	80	60
N798.S	2	60	30	2	80	40
N777.S	2	20	30	2	80	50
N968.S	2	70	0	2	80	30
N827.S	2	40	70	2	70	90
N464.S	2	40	40	2	70	50
N665.S	2	0	10	2	60	20
N1089	2	20	60	2	50	60
N832.S	2	50	0	2	50	0
N703.S	2	40	60	2	40	70
N1093	2	40	30	2	40	30
N660.S	2	0	0	2	40	50
N815.S	2	10	0	2	30	10
N1019.S	2	0	30	2	20	30
N1002.S	2	20	10	2	20	10
N685.S	2	20	10	2	20	10
N739.S	2	20	10	2	20	10
N835.S	2	20	10	2	20	10
N842.S	2	20	10	2	20	10
N843.S	2	20	10	2	20	10
N960.S	2	20	10	2	20	10
N995.S	2	20	10	2	20	10
N741.S	2	10	10	2	20	10
N795.S	2	10	10	2	20	10
N945.S	2	10	10	2	20	10
N796.S	2	0	10	2	20	10
N668.S	2	20	0	2	20	0
N891.S	2	20	0	2	20	0
N928.S	2	20	0	2	20	0
N936.S	2	20	0	2	20	0
N667.S	2	0	0	2	20	10
N826.S	2	0	0	2	20	10
N822.S	2	10	10	2	10	10

Network Ecology ID	Number of OTUs	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network	Number of Clades	Percent of Ethanol treated spore Preparations Containing Network	Percent of Combined Augmented & Engrafted Post Treatment Patient Ecologies Containing Network
N733.S	2	0	10	2	10	10
N816.S	2	0	10	2	10	10
N1004.S	2	10	0	2	10	0
N805.S	2	10	0	2	10	0
N809.S	2	10	0	2	10	0
N851.S	2	10	0	2	10	0
N1078.S	2	0	0	2	10	0
N789.S	2	0	0	2	10	0
N800.S	2	0	0	2	10	0
N808.S	2	0	0	2	10	0
N811.S	2	0	0	2	10	0
N1091	2	0	10	2	0	10
N782.S	2	0	10	2	0	10
N776.S	2	0	0	2	0	10

Table 15.

Presence of Keystone OTU Clades in ethanol-treated spore population treatment and patient's pretreatment, augmented and engrafted microbial ecologies post-treatment.

Keystone clade	Percent of Augmented Ecologies with clade	Percent of Engrafted Ecologies with clade	Percent of EcoM Purified Spore Treatment Ecologies with clade	Percent of Pretreatment Ecologies with clade
clade_65	20	20	40	20
clade_85	0	0	0	0
clade_98	40	30	100	80
clade_110	10	0	10	0
clade_170	10	0	30	0
clade_172	10	50	100	30
clade_262	50	80	100	30
clade_262	50	80	100	30
clade_286	0	20	60	20
clade_286	0	20	60	20
clade_309	40	100	100	40
clade_358	40	0	20	80
clade_360	20	100	100	60
clade_360	20	100	100	60
clade_378	10	30	80	0
clade_393	10	90	100	10
clade_396	70	90	100	10
clade_408	60	100	100	50
clade_444	70	90	100	10
clade_466	10	0	0	0
clade_478	0	90	100	30
clade_485	60	10	40	10
clade_494	50	80	100	30
clade_500	10	0	20	10
clade_521	0	0	100	30
clade_537	50	90	100	20
clade_538	10	0	0	0
clade_540	90	10	30	10
clade_543	10	90	100	0
clade_572	30	80	100	20
clade_576	50	30	50	30
clade_583	20	10	60	50

Table 16.

Efficacy of Network Ecologies screened in *Clostridium difficile* Infection Prevention Mouse Model.

SP Expt.	SP Arm	Test Article	Target Dose (CFU/CFU /mouse)	Cumulative Mortality (%)	Mean Min. Rel Weight	Mean Max. Clin. Score (Death = 4)
SP-327	3	Vehicle Control		30	0.89	2.2
SP-327	4	Vanco. Positive Control		0	0.99	1
SP-327	12	N1957	2.0E+07	0	0.87	0
SP-327	13	N1957	2.0E+06	40	0.86	2.2
SP-327	14	N1957	2.0E+05	50	0.80	2.8
SP-338	1	Vehicle Control		60	0.81	3.2
SP-338	2	Vanco. Positive Control		0	1.00	0
SP-338	3	10% fecal suspension		0	0.95	1
SP-338	5	N1957	2.0E+07	10	0.80	2
SP-338	6	N1957	2.0E+06	0	0.97	1
SP-338	7	N1957	2.0E+05	20	0.85	1.7
SP-338	11	N1957	2.0E+07	20	0.86	2
SP-338	12	N1957	2.0E+06	30	0.83	2.5
SP-338	13	N1961	2.0E+07	10	0.93	1.3
SP-338	14	N1955	2.0E+07	0	0.91	1.2
SP-338	15	N1955	2.0E+06	10	0.90	1.5
SP-338	16	N1955	2.0E+05	10	0.89	2.7
SP-338	17	N1967	2.0E+07	10	0.94	1.4
SP-338	18	N1983	2.0E+07	0	0.92	1
SP-338	19	N1989	2.0E+07	10	0.91	1.3
SP-338	20	N1996	2.0E+07	10	0.93	1.3
SP-338	21	Naive		0	1.00	0
SP-339	1	Vehicle Control		20	0.88	2.2
SP-339	2	Vanco. Positive Control		0	0.99	0
SP-339	3	10% fecal suspension		0	0.97	0
SP-339	4	N1995	2.0E+07	20	0.83	2.1
SP-339	5	N1995	2.0E+06	10	0.91	1.5
SP-339	6	N1995	2.0E+05	0	0.96	1.2
SP-339	7	N1950	2.0E+07	0	0.94	1
SP-339	8	N1994	2.0E+07	20	0.87	1.8
SP-339	9	N1997	2.0E+07	0	0.95	1.2
SP-339	10	N1967	2.0E+07	0	0.93	1.2
SP-339	11	N1983	2.0E+07	10	0.83	2.2
SP-339	12	N1989	2.0E+07	0	0.88	1.5
SP-339	13	N1996	2.0E+07	0	0.97	1
SP-339	14	N2002	2.0E+07	20	0.92	2
SP-339	15	N2000	2.0E+07	0	0.98	1.2
SP-339	21	Naive		0	0.98	0
SP-342	1	Vehicle Control		40	0.85	2.5
SP-342	2	Vanco. Positive Control		0	1.00	0
SP-342	5	N1957	2.0E+08	0	0.94	0.2

SP Expt.	SP Arm	Test Article	Target Dose (CFU/0.2 mL)	Cumulative Mortality (%)	Mean Min. Rel. Weight	Mean Max. Clin. Score (Study Day)
SP-342	6	N1957	2.0E+07	0	0.96	0
SP-342	7	N1957	2.0E+06	10	0.88	1.3
SP-342	8	N1980	2.0E+08	10	0.92	1.8
SP-342	9	N1998	2.0E+08	20	0.83	2.8
SP-342	10	N1976	2.0E+08	10	0.92	1.4
SP-342	11	N1987	2.0E+08	10	0.93	1.6
SP-342	12	N2005	2.0E+08	20	0.86	2.4
SP-342	13	N1958	2.0E+08	0	0.94	1.5
SP-342	14	N2004	2.0E+08	10	0.93	1.4
SP-342	15	N1949	2.0E+08	10	0.87	1.5
SP-342	18	N1970	2.0E+08	50	0.81	3
SP-342	21	Naïve		0	0.99	0
SP-361	1	Vehicle Control		30	0.88	2.6
SP-361	2	10% fecal suspension		0	0.99	0
SP-361	3	N435	1.0E+07	80	0.83	3.6
SP-361	4	N1979	1.0E+07	0	0.97	0
SP-361	5	N414	1.0E+07	0	0.97	0
SP-361	6	N512	1.0E+07	20	0.94	1.6
SP-361	7	N582	1.0E+07	10	0.93	0.9
SP-361	8	N571	1.0E+07	30	0.88	2.1
SP-361	9	N510	1.0E+07	0	0.93	0.3
SP-361	10	N1981	1.0E+07	40	0.83	2.8
SP-361	11	N1969	1.0E+07	80	0.82	3.6
SP-361	12	N461	1.0E+07	10	0.89	1.2
SP-361	13	N460	1.0E+07	0	0.93	1.1
SP-361	14	N1959	1.0E+07	30	0.89	1.9
SP-361	15	N2006	1.0E+07	30	0.89	1.9
SP-361	16	N1953	1.0E+07	10	0.83	2.3
SP-361	17	N1960	1.0E+07	0	0.92	1
SP-361	18	N2007	1.0E+07	10	0.91	0.9
SP-361	19	N1978	1.0E+07	10	0.91	1.3
SP-361	20	N1972	1.0E+07	30	0.83	2.6
SP-361	21	Naïve		0	1.00	0
SP-363	1	Vehicle Control		30	0.85	2.6
SP-363	2	10% fecal suspension		0	0.95	0
SP-363	8	N1974	1.0E+07	60	0.81	3.2
SP-363	9	N582	1.0E+07	60	0.81	3.2
SP-363	10	N435	1.0E+07	30	0.86	2.1
SP-363	11	N414	1.0E+07	40	0.83	2.5
SP-363	12	N457	1.0E+07	30	0.83	2.2
SP-363	13	N511	1.0E+07	20	0.87	2
SP-363	14	N513	1.0E+07	0	0.88	0.2
SP-363	15	N682	1.0E+07	30	0.82	2.6

SP Expt.	SP Arm	Test Article	Target Dose (CFU/0.2 mL)	Cumulative Mortality (%)	Mean Min. Rel. Weight	Mean Max. Clin. Score (Day(s) 1-4)
SP-363	16	N736	1.0E+07	40	0.82	2.8
SP-363	17	N732	1.0E+07	10	0.86	1.3
SP-363	18	N1948	1.0E+07	60	0.85	3.2
SP-363	19	N853	1.0E+07	10	0.85	2.2
SP-363	20	N1979	1.0E+07	60	0.78	3.2
SP-363	21	N879	1.0E+07	40	0.83	2.8
SP-363	22	N999	1.0E+07	20	0.88	2.4
SP-363	23	N975	1.0E+07	30	0.80	2.6
SP-363	24	N861	1.0E+07	50	0.85	3
SP-363	25	N1095	1.0E+07	80	0.83	3.6
SP-363	26	Naïve		0	1.00	0
SP-364	1	Vehicle Control		40	0.83	2.8
SP-364	4	N582	1.0E+07	0	0.81	0.9
SP-364	5	N582	1.0E+06	0	0.84	0.9
SP-364	6	N582	1.0E+05	40	0.76	2.5
SP-364	13	N414	1.0E+07	0	0.84	0
SP-364	14	N414	1.0E+06	30	0.79	2.4
SP-364	15	N414	1.0E+05	10	0.76	2
SP-364	22	10% fecal suspension		0	0.97	0
SP-364	23	Naïve		0	0.99	0
SP-365	1	Vehicle Control		40	0.83	2.8
SP-365	4	10% fecal suspension		0	0.98	0
SP-365	13	N582	1.0E+07	60	0.80	3.2
SP-365	14	N582	1.0E+06	10	0.89	1.5
SP-365	15	N414	1.0E+07	20	0.86	1.7
SP-365	16	N414	1.0E+06	80	0.83	3.5
SP-365	21	Naïve		0	1.00	0
SP-366	1	Vehicle Control		20	0.82	2.4
SP-366	4	10% fecal suspension		0	0.93	1
SP-366	7	N582	1.0E+07	0	0.86	1
SP-366	10	N414	1.0E+07	20	0.83	2.4
SP-366	13	N402	1.0E+07	30	0.81	2.1
SP-366	16	N1982	1.0E+07	0	0.90	1.1
SP-366	19	N460	1.0E+07	10	0.83	2.2
SP-366	22	N513	6.7E+06	40	0.82	2.8
SP-366	23	N1966	1.0E+07	0	0.90	0.5
SP-366	24	N1977	1.0E+07	20	0.83	1.9
SP-366	25	N1979	1.0E+07	20	0.83	2.4
SP-366	26	N682	1.0E+07	20	0.83	2.3
SP-366	27	N1947	1.0E+07	10	0.82	1.3
SP-366	28	N582	1.0E+07	20	0.82	1.8
SP-366	29	N414	1.0E+07	0	0.85	1.5
SP-366	30	N603	1.0E+07	30	0.82	2.2

SP Expt.	SP Arm	Test Article	Target Dose (CFU/ml)	Cumulative Mortality (%)	Mean Min. Rel. Weight	Mean Max. Clin. Score (Study Day)
SP-366	31	Naïve		0	0.99	0
SP-368	1	Vehicle Control		50	0.85	2.8
SP-368	2	10% fecal suspension		0	0.97	0
SP-368	3	Ethanol-treated fecal spores A		20	0.90	1.8
SP-368	7	N1966	1.0E+07	0	0.89	1
SP-368	8	N1966	1.0E+06	10	0.91	1.5
SP-368	9	N1966	1.0E+05	50	0.82	3.1
SP-368	10	Ethanol-treated fecal spores B		0	0.99	0
SP-368	15	Ethanol-treated fecal spores C		0	0.95	1
SP-368	21	Naïve		0	1.00	0
SP-374	1	Vehicle Control		100	0.83	4
SP-374	4	10% fecal suspension		10	0.89	0.5
SP-374	11	N1966	1.0E+08	0	0.87	1
SP-374	12	N1966	1.0E+08	0	0.91	0.5
SP-374	13	N1966	1.0E+07	10	0.88	1.3
SP-374	14	N1966	1.0E+06	50	0.79	3
SP-374	15	N584	1.0E+08	0	0.89	1
SP-374	16	N584	1.0E+07	30	0.84	2.4
SP-374	17	N1962	1.0E+07	0	0.93	0
SP-374	18	N382	1.0E+07	10	0.85	1.5
SP-374	19	N1964	1.0E+07	20	0.89	1.8
SP-374	20	N1965	1.0E+07	30	0.85	2.1
SP-374	21	N306	1.0E+07	10	0.90	0.4
SP-374	22	N1988	1.0E+07	0	0.89	1
SP-374	23	N2003	1.0E+07	0	0.92	1.2
SP-374	24	N1993	1.0E+07	20	0.77	2.4
SP-374	25	Naïve		0	0.99	0
SP-376	1	Vehicle Control		60	0.83	3.2
SP-376	2	10% fecal suspension		0	0.98	0
SP-376	3	N1966	1.0E+08	30	0.79	2.4
SP-376	4	N1966	1.0E+07	0	0.95	0
SP-376	5	N1966	1.0E+08	30	0.79	2.6
SP-376	6	N1966	1.0E+07	10	0.88	2.2
SP-376	7	N1986	1.0E+07	40	0.80	2.8
SP-376	8	N1962	1.0E+08	0	0.98	0
SP-376	9	N1962	1.0E+07	0	0.95	0
SP-376	10	N1963	1.0E+07	40	0.81	2.6
SP-376	11	N1984	1.0E+08	0	0.97	0
SP-376	12	N1984	1.0E+07	0	0.90	1.1
SP-376	13	N1990	1.0E+08	0	0.92	1
SP-376	14	N1990	1.0E+07	0	0.92	1
SP-376	15	N1999	1.0E+08	10	0.87	1.4
SP-376	16	N1999	1.0E+07	0	0.93	0

SP Expt	SP Arm	Test Article	Target Dose (CFU/0.2 mL) (mean)	Cumulative Mortality (%)	Mean Min. Rel. Weight	Mean Max. Clin. Score (Study Day)
SP-376	17	N1968	1.0E+07	50	0.78	3
SP-376	18	N1951	1.0E+07	0	0.93	1
SP-376	19	N1991	1.0E+07	0	0.93	1.1
SP-376	20	N1975	1.0E+07	50	0.78	3
SP-376	21	Naïve		0	0.99	0
SP-383	1	Vehicle Control		100	0.83	4
SP-383	2	10% fecal suspension		0	0.92	0.1
SP-383	9	N1962	1.0E+09	10	0.95	1.3
SP-383	10	N1962	1.0E+08	10	0.93	1.3
SP-383	11	N1962	1.0E+07	0	0.92	1
SP-383	12	N1984	1.0E+09	0	0.89	1
SP-383	13	N1984	1.0E+08	10	0.94	1.3
SP-383	14	N1984	1.0E+07	10	0.90	1.3
SP-383	21	Naïve		0	1.00	0
SP-390	1	Vehicle Control		80	0.82	3.6
SP-390	2	10% fecal suspension		0	0.98	0.1
SP-390	3	N1962	2.0E+07	0	0.97	0
SP-390	4	N1962	2.0E+06	0	0.98	0
SP-390	5	N1984	2.0E+07	0	0.95	1
SP-390	6	N1984	2.0E+06	0	0.95	0.1
SP-390	9	N1962	2.0E+07	0	0.93	1
SP-390	10	N1962	2.0E+06	10	0.93	1.3
SP-390	11	N1984	2.0E+07	20	0.86	2.2
SP-390	12	N1984	2.0E+06	30	0.88	2.1
SP-390	13	N1952	2.0E+07	0	0.89	1
SP-390	14	N2001	2.0E+07	0	0.95	0.2
SP-390	15	N1973	2.0E+07	10	0.90	0.7
SP-390	16	N1954	2.0E+07	0	0.94	1.1
SP-390	17	N1985	2.0E+07	10	0.86	1.8
SP-390	18	N1971	2.0E+07	0	0.89	0.9
SP-390	19	N1956	2.0E+07	0	0.95	0
SP-390	20	N1992	2.0E+07	0	0.95	0
SP-390	31	Naïve		0	0.98	0

Table 17.

Network Ecologies screened *in vivo* in Clostridium difficile Infection Prevention Mouse Model

Network Ecology ID	Exemplary Network Clades	Exemplary Network OTUs	Exemplary Keystone OTUs
N306	clade_252, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_38 or clade_38e or clade_38i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), clade_494, (clade_553 or clade_553i)	Blautia producta, Clostridium hylemonae, Clostridium innocuum, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprobacillus sp. D7, Coprococcus comes, Eubacterium rectale, Eubacterium sp. WAL 14571, Faecalibacterium prausnitzii, Lachnospiraceae bacterium 5_1_57FAA, Roseburia faecalis, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N382	clade_252, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium hylemonae, Clostridium innocuum, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N402	(clade_262 or clade_262i), clade_286, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i), clade_500	Alistipes shahii, Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Parabacteroides merdae, Ruminococcus obeum, Ruminococcus torques	Alistipes shahii, Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Parabacteroides merdae, Ruminococcus obeum, Ruminococcus torques
N414	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i), (clade_522 or clade_522i)	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum, Ruminococcus torques
N435	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum, Ruminococcus	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum, Ruminococcus

	clade_444i), clade_466, (clade_478 or clade_478i)	torques	torques
N457	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_522 or clade_522i)	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques
N460	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i)	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum, Ruminococcus torques
N461	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N510	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_522 or clade_522i)	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N511	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques
N512	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i)	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Ruminococcus obeum, Ruminococcus torques
N513	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques

N571	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N582	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i)	Clostridium symbiosum, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N584	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_553 or clade_553i)	Blautia producta, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA	Coprococcus comes
N603	(clade_172 or clade_172i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Bifidobacterium adolescentis, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum
N682	clade_170, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Bacteroides caccae, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum	Bacteroides caccae, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum
N732	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_478 or clade_478i)	Coprococcus comes, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N736	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_478 or clade_478i)	Dorea longicatena, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Dorea longicatena, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N853	(clade_262 or clade_262i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques	Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus torques
N861	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h)	Clostridium hathewayi, Ruminococcus obeum, Ruminococcus torques	Ruminococcus obeum, Ruminococcus torques

N879	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_444 or clade_444i), (clade_478 or clade_478i)	Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum	Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum
N975	clade_170, (clade_262 or clade_262i), (clade_360 or clade_360c or clade_360g or clade_360h clade_360i)	Bacteroides caccae, Coprococcus comes, Dorea longicatena	Bacteroides caccae, Coprococcus comes, Dorea longicatena
N999	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_478 or clade_478i)	Dorea formicigenerans, Faecalibacterium prausnitzii, Ruminococcus obeum	Dorea formicigenerans, Faecalibacterium prausnitzii, Ruminococcus obeum
N1095	(clade_444 or clade_444i), (clade_522 or clade_522i)	Eubacterium eligens, Eubacterium rectale	Eubacterium eligens, Eubacterium rectale
N1947	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_38 or clade_38e or clade_38i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_553 or clade_553i), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 3_1_23, Collinsella aerofaciens, Dorea longicatena, Escherichia coli, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques	Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques
N1948	(clade_262 or clade_262i), (clade_38 or clade_38e or clade_38i), (clade_478 or clade_478i), (clade_65 or clade_65e)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Faecalibacterium prausnitzii, Ruminococcus torques	Faecalibacterium prausnitzii, Ruminococcus torques
N1949	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Enterococcus faecalis, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli	
N1950	clade_253, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_65 or clade_65e)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium disporicum, Erysipelotrichaceae bacterium 3_1_53	
N1951	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or	Blautia producta, Clostridium bolteae, Clostridium hylemonae, Clostridium symbiosum, Coprococcus comes, Eubacterium rectale, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus gnavus	Coprococcus comes, Eubacterium rectale

	clade_444i)		
N1952	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N1953	clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides vulgatus, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Coprobacillus sp. D7, Coprococcus comes, Dorea formicigenerans, Enterococcus faecalis, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum
N1954	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia sp. M25, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Blautia sp. M25, Coprococcus comes, Ruminococcus bromii

N1955	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 2_1_22, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Citrobacter sp. 30_2, Clostridium sordellii, Coprobacillus sp. D7, Enterococcus faecalis, Enterococcus faecium, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli	
N1956	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium nexile, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Clostridium nexile, Ruminococcus bromii
N1957	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium innocuum, Clostridium sordellii, Coprobacillus sp. D7, Enterococcus faecalis, Escherichia coli	
N1958	(clade_351 or clade_351e), (clade_354 or clade_354e), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i)	Clostridium innocuum, Clostridium sordellii, Coprobacillus sp. D7	
N1959	clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_522 or	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium disporicum, Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Enterococcus faecalis, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques

	clade_522i), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)		
N1960	clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_92 or clade_92e or clade_92i)	Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Coprobacillus sp. D7, Coprococcus comes, Dorea formicigenerans, Enterococcus faecalis, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum
N1961	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium innocuum, Enterococcus faecalis, Escherichia coli	
N1962	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii

N1963	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N1964	clade_170, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), clade_286, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), clade_485, clade_500, clade_537, (clade_553 or clade_553i), clade_85	Alistipes shahii, Bacteroides caccae, Bacteroides stercoris, Blautia producta, Clostridium hathewayi, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Holdemania filiformis, Lachnospiraceae bacterium 5_1_57FAA, Parabacteroides merdae, Ruminococcus bromii, Ruminococcus obeum, Ruminococcus torques	Alistipes shahii, Bacteroides caccae, Bacteroides stercoris, Coprococcus comes, Eubacterium rectale, Holdemania filiformis, Parabacteroides merdae, Ruminococcus bromii, Ruminococcus obeum, Ruminococcus torques
N1965	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Lachnospiraceae bacterium 5_1_57FAA, Roseburia intestinalis, Ruminococcus bromii, Ruminococcus obeum	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Ruminococcus bromii, Ruminococcus obeum
N1966	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA	Coprococcus comes
N1967	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Enterococcus faecium, Escherichia coli	

N1968	clade_252, clade_253, (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_478 or clade_478i), clade_494, clade_537, (clade_553 or clade_553i)	Clostridium butyricum, Clostridium disporicum, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium tertium, Collinsella aerofaciens, Faecalibacterium prausnitzii, Ruminococcus bromii	Faecalibacterium prausnitzii, Ruminococcus bromii
N1969	clade_252, clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_479 or clade_479c or clade_479g or clade_479h)	Blautia producta, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Dorea formicigenerans, Erysipelotrichaceae bacterium 3_1_53, Ruminococcus torques	Dorea formicigenerans, Ruminococcus torques
N1970	(clade_351 or clade_351e), (clade_354 or clade_354e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Clostridium innocuum, Clostridium sordellii, Coprobacillus sp. D7, Enterococcus faecalis, Escherichia coli	
N1971	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Blautia schinkii, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N1972	clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i)	Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Coprobacillus sp. D7, Coprococcus comes, Dorea formicigenerans, Erysipelotrichaceae bacterium 3_1_53, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum

N1973	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N1974	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i)	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques
N1975	(clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus bromii
N1976	(clade_351 or clade_351e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Clostridium innocuum, Coprobacillus sp. D7, Enterococcus faecalis	
N1977	clade_252, clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_479 or clade_479c or clade_479g or clade_479h)	Blautia producta, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Dorea formicigenerans, Erysipelotrichaceae bacterium 3_1_53, Eubacterium tenue, Ruminococcus torques	Dorea formicigenerans, Ruminococcus torques

N1978	clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides vulgatus, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Coprobacillus sp. D7, Coprococcus comes, Dorea formicigenerans, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum
N1979	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_65 or clade_65e)	Bacteroides sp. 1_1_6, Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N1980	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_92 or clade_92e or clade_92i)	Blautia producta, Clostridium innocuum, Clostridium sordellii, Coprobacillus sp. D7, Enterococcus faecalis, Escherichia coli	
N1981	(clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_38 or clade_38e or clade_38i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_522 or clade_522i)	Bacteroides sp. 3_1_23, Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Dorea longicatena, Eubacterium eligens, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques

N1982	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Dorea formicigenerans, Erysipelotrichaceae bacterium 3_1_53, Eubacterium rectale, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus obeum, Ruminococcus torques	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Ruminococcus obeum, Ruminococcus torques
N1983	clade_252, clade_253, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Enterococcus faecium, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli	
N1984	clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), clade_494, (clade_553 or clade_553i)	Blautia producta, Clostridium disporicum, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Collinsella aerofaciens, Eubacterium rectale, Lachnospiraceae bacterium 5_1_57FAA	Eubacterium rectale
N1985	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia glucerasei, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii

N1986	clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_553 or clade_553i)	Blautia producta, Clostridium disporicum, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA	Coprococcus comes
N1987	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_92 or clade_92e or clade_92i)	Blautia producta, Clostridium sordellii, Escherichia coli	
N1988	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_485, clade_500, (clade_553 or clade_553i)	Alistipes shahii, Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Holdemania filiformis, Lachnospiraceae bacterium 5_1_57FAA, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques	Alistipes shahii, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Holdemania filiformis, Roseburia intestinalis, Ruminococcus obeum, Ruminococcus torques
N1989	clade_252, clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Dorea formicigenerans, Enterococcus faecium, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli, Eubacterium tenue, Ruminococcus torques	Dorea formicigenerans, Ruminococcus torques
N1990	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus bromii

N1991	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus gnavus	Coprococcus comes, Eubacterium rectale
N1992	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Lachnospiraceae bacterium 1_4_56FAA, Ruminococcus bromii
N1993	clade_110, clade_170, (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_65 or clade_65e), clade_85	Bacteroides caccae, Bacteroides eggerthii, Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides stercoris, Bacteroides uniformis, Bacteroides vulgatus	Bacteroides caccae, Bacteroides eggerthii, Bacteroides stercoris, Bacteroides uniformis
N1994	clade_253, (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Clostridium disporicum, Enterococcus faecium, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli	
N1995	clade_253, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Clostridium disporicum, Enterococcus faecium, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli	
N1996	clade_253, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_479 or clade_479c or clade_479g or clade_479h)	Blautia producta, Clostridium disporicum, Erysipelotrichaceae bacterium 3_1_53	

N1997	clade_253, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_497 or clade_497e or clade_497f), (clade_92 or clade_92e or clade_92i)	Blautia producta, Clostridium disporicum, Enterococcus faecium, Erysipelotrichaceae bacterium 3_1_53, Escherichia coli	
N1998	(clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_65 or clade_65e)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus	
N1999	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii, Lachnospiraceae bacterium 5_1_57FAA	Coprococcus comes, Eubacterium rectale, Faecalibacterium prausnitzii
N2000	clade_252, clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_479 or clade_479c or clade_479g or clade_479h)	Blautia producta, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Dorea formicigenerans, Erysipelotrichaceae bacterium 3_1_53, Eubacterium tenue, Ruminococcus torques	Dorea formicigenerans, Ruminococcus torques
N2001	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_351 or clade_351e), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), clade_494, clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium disporicum, Clostridium hylemonae, Clostridium innocuum, Clostridium mayombei, Clostridium orbiscindens, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprococcus comes, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N2002	clade_252, clade_253, (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_479 or clade_479c or clade_479g or clade_479h)	Blautia producta, Clostridium butyricum, Clostridium disporicum, Clostridium mayombei, Erysipelotrichaceae bacterium 3_1_53	

N2003	clade_252, clade_253, (clade_260 or clade_260c or clade_260g or clade_260h), (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_38 or clade_38e or clade_38i), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), clade_537, (clade_553 or clade_553i)	Blautia producta, Clostridium bolteae, Clostridium butyricum, Clostridium disporicum, Clostridium hylemonae, Clostridium mayombei, Clostridium sordellii, Clostridium symbiosum, Clostridium tertium, Collinsella aerofaciens, Coprobacillus sp. D7, Coprococcus comes, Eubacterium sp. WAL 14571, Lachnospiraceae bacterium 5_1_57FAA, Ruminococcus bromii, Ruminococcus gnavus	Coprococcus comes, Ruminococcus bromii
N2004	(clade_351 or clade_351e), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Clostridium innocuum, Enterococcus faecalis, Escherichia coli	
N2005	(clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_378 or clade_378e), (clade_38 or clade_38e or clade_38i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e), (clade_92 or clade_92e or clade_92i)	Bacteroides sp. 1_1_6, Bacteroides sp. 3_1_23, Bacteroides vulgatus, Blautia producta, Enterococcus faecalis, Escherichia coli	
N2006	clade_170, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_444 or clade_444i), (clade_478 or clade_478i), (clade_65 or clade_65e)	Bacteroides caccae, Bacteroides sp. 1_1_6, Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques	Bacteroides caccae, Coprococcus comes, Dorea formicigenerans, Dorea longicatena, Eubacterium rectale, Faecalibacterium prausnitzii, Ruminococcus obeum, Ruminococcus torques
N2007	clade_253, (clade_262 or clade_262i), (clade_309 or clade_309c or clade_309e or clade_309g or clade_309h or clade_309i), (clade_354 or clade_354e), (clade_360 or clade_360c or clade_360g or clade_360h or clade_360i), (clade_378 or clade_378e), (clade_408 or clade_408b or clade_408d or clade_408f or clade_408g or clade_408h), (clade_444 or clade_444i), clade_466, (clade_478 or clade_478i), (clade_479 or clade_479c or clade_479g or clade_479h), (clade_481 or clade_481a or clade_481b or clade_481e or clade_481g or clade_481h or clade_481i), (clade_497 or clade_497e or clade_497f), (clade_65 or clade_65e)	Bacteroides sp. 1_1_6, Bacteroides vulgatus, Clostridium disporicum, Clostridium mayombei, Clostridium symbiosum, Coprobacillus sp. D7, Coprococcus comes, Dorea formicigenerans, Enterococcus faecalis, Erysipelotrichaceae bacterium 3_1_53, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum	Coprococcus comes, Dorea formicigenerans, Eubacterium rectale, Faecalibacterium prausnitzii, Odoribacter splanchnicus, Ruminococcus obeum

Table 18

KEGG Orthology Pathway (KO) characterization and F-score of computationally determined network ecologies reported in Table 8 that represent states of health for multiple disease indications.

Net ID	F Score	KEGG Orthology Pathways
N253	29	KO:K00004, KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K01908, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N250	29	KO:K00004, KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K01908, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N249	29	KO:K00004, KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K01908, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N252	29	KO:K00004, KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K01908, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N251	28	KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K01908, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N257	27	KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N254	27	KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N255	27	KO:K00023, KO:K00101, KO:K00102, KO:K00116, KO:K00156, KO:K00158, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01720, KO:K01734, KO:K01905, KO:K01907, KO:K03366, KO:K03416, KO:K03417, KO:K03777, KO:K03778, KO:K03821, KO:K04020, KO:K05973
N1036	17	KO:K00101, KO:K00116, KO:K00156, KO:K00163, KO:K00627, KO:K00656, KO:K00932, KO:K01069, KO:K01442, KO:K01659, KO:K01720, KO:K01734, KO:K01908, KO:K03417, KO:K03777, KO:K03778, KO:K04020
N968	17	KO:K00101, KO:K00116, KO:K00156, KO:K00163, KO:K00627, KO:K00656, KO:K00932, KO:K01069, KO:K01442, KO:K01659, KO:K01720, KO:K01734, KO:K01908, KO:K03417, KO:K03777, KO:K03778, KO:K04020
N335	15	KO:K00102, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778, KO:K13923
N329	15	KO:K00102, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778, KO:K13923
N365	13	KO:K00004, KO:K00116, KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N363	13	KO:K00004, KO:K00116, KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N282	13	KO:K00004, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N280	13	KO:K00004, KO:K00116, KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N306	13	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03417, KO:K03778, KO:K07246
N623	12	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K13923
N350	12	KO:K00102, KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N324	12	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N914	12	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K13923
N351	12	KO:K00102, KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N296	12	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N917	12	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K13923
N299	12	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N790	12	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K13923
N386	12	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N275	12	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N777	12	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K13923
N318	12	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N271	12	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N816	12	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N256	12	KO:K00102, KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N259	12	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K07246
N262	12	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N382	12	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K01905, KO:K03417, KO:K03778, KO:K07246
N424	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N640	11	KO:K00102, KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734,

		KO:K03778, KO:K04020
N505	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N590	11	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N641	11	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N625	11	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N410	11	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K07246
N586	11	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N326	11	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01734, KO:K03778
N522	11	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K01896, KO:K03417, KO:K03778, KO:K07246
N265	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N588	11	KO:K00102, KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N1074	11	KO:K00004, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N610	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N408	11	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K07246
N548	11	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N383	11	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N269	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N901	11	KO:K00004, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N419	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N443	11	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01734, KO:K03778
N470	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N908	11	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N631	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N619	11	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N926	11	KO:K00004, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778

N429	11	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01659, KO:K01734, KO:K03778
N390	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N368	11	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N345	11	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N354	11	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N825	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N822	11	KO:K00004, KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N573	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N666	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N288	11	KO:K00156, KO:K00158, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N650	11	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N338	11	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N381	11	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N292	11	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N818	11	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N516	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N515	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N276	11	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N258	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N851	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01734, KO:K03778, KO:K04020, KO:K07246
N307	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N400	11	KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N361	10	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N558	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N891	10	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K01905, KO:K03778,

		KO:K07246
N904	10	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N274	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N612	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N501	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N643	10	KO:K00156, KO:K00158, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N633	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N866	10	KO:K00102, KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K01905, KO:K03778
N444	10	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01734, KO:K03778
N535	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N552	10	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1072	10	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K01905, KO:K03778, KO:K07246
N597	10	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N539	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N717	10	KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N471	10	KO:K00156, KO:K00158, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N268	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N298	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N409	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N489	10	KO:K00156, KO:K00158, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N266	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N319	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N566	10	KO:K00043, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
N542	10	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N337	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N886	10	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N528	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N295	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N966	10	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N940	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N407	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N481	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N336	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N530	10	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N567	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N525	10	KO:K00043, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
N771	10	KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N644	10	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N560	10	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N595	10	KO:K00156, KO:K00158, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N349	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N531	10	KO:K00004, KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N568	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N707	10	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N366	10	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N797	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N791	10	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N375	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N387	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N346	10	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734,

		KO:K03778
N342	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N834	10	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N314	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N332	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N352	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N367	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N333	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N519	10	KO:K00004, KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N353	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N680	10	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N283	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N264	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N281	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N821	10	KO:K00156, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N273	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N260	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N817	10	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N438	10	KO:K00116, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01734, KO:K03778
N263	10	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N261	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N313	10	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N401	10	KO:K00102, KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K04020
N584	10	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N286	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N426	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N362	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N270	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N716	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
N277	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N614	9	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N423	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N311	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N546	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1073	9	KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
N635	9	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N492	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N770	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
N422	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N882	9	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K04020
N427	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N700	9	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1030	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N450	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N322	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N308	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N549	9	KO:K00004, KO:K00116, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N404	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1070	9	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734
N601	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N954	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N699	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N884	9	KO:K00156, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N309	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N488	9	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N496	9	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N364	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N502	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N297	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N618	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N310	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N526	9	KO:K00004, KO:K00116, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N1083	9	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N749	9	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K04020
N608	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N555	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N955	9	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N454	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N622	9	KO:K00004, KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N477	9	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N497	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N325	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N285	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N534	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N896	9	KO:K00116, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01505, KO:K01734
N1008	9	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N465	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N328	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N646	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N449	9	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N320	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N445	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1097	9	KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N384	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N898	9	KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
N406	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N536	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N321	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N965	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K07246
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N415	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N480	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N478	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N701	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N547	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N945	8	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N762	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N493	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N739	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N912	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N484	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N720	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1014	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N744	8	KO:K00156, KO:K00656, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N451	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N565	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N498	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N483	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N628	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N889	8	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N624	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N731	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1038	8	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N727	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N615	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N697	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N538	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N688	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1037	8	KO:K00163, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N541	8	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N721	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N1096	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N629	8	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N504	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N587	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N476	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N564	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N746	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N490	8	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

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N693	8	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N559	8	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N712	8	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N467	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N711	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N405	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N524	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N593	8	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N941	8	KO:K00102, KO:K00156, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N752	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1019	8	KO:K00156, KO:K00656, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N503	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N455	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N485	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1042	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N684	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
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N792	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N661	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N462	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N672	8	KO:K00156, KO:K00163, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N664	8	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
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N674	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N579	8	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N795	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N430	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N416	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N794	8	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N651	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1091	8	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734

N1093	8	KO:K00156, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N437	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N683	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N958	7	KO:K00156, KO:K00656, KO:K01067, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N907	7	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03366
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N906	7	KO:K00102, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N745	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N952	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N723	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N611	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N875	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N1005	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N975	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N992	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N936	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N913	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01734, KO:K03417, KO:K07246
N994	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N685	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N1054	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N997	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N760	7	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N532	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1039	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N1041	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N944	7	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N585	7	KO:K00163, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N754	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N696	7	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N887	7	KO:K00156, KO:K00163, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734
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N491	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N540	7	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N903	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N888	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N468	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N695	7	KO:K00116, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
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N507	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N894	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
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N613	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N520	7	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
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N580	7	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
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N537	6	KO:K00102, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
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N764	6	KO:K00158, KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N874	6	KO:K00163, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N600	6	KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N710	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778

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N557	6	KO:K00102, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
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N809	6	KO:K00656, KO:K01069, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N858	6	KO:K00043, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N508	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N681	6	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N845	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N673	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N521	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1095	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N581	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N827	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N570	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N571	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N574	6	KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N779	6	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N578	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N457	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N652	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N828	6	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734
N514	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N653	6	KO:K00043, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N849	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N436	6	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N853	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N786	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N512	6	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N510	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N861	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N647	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1071	5	KO:K00043, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N932	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N750	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N893	5	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N897	5	KO:K00043, KO:K00627, KO:K00656, KO:K01442, KO:K01734
N729	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1003	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1069	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N616	5	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N964	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N920	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N1061	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N738	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N983	5	KO:K00627, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N757	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N755	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1004	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N879	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N962	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N1016	5	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01734
N533	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N645	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N736	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N713	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1006	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N925	5	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01734
N620	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N880	5	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N747	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N885	5	KO:K00043, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N737	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N554	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N951	5	KO:K00102, KO:K00656, KO:K01069, KO:K01734, KO:K03778
N690	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N730	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N999	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N589	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N545	5	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N763	5	KO:K00656, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N1067	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N703	5	KO:K00656, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N773	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N756	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K10783
N1062	5	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N1046	5	KO:K00627, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N742	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N704	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N751	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N939	5	KO:K00627, KO:K00656, KO:K01069, KO:K01734, KO:K03778
N984	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N599	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N1066	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N689	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K10783
N562	5	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N918	5	KO:K00656, KO:K01069, KO:K01734, KO:K03778, KO:K10783
N1078	5	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01734
N1007	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N637	5	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N859	5	KO:K00102, KO:K00656, KO:K01069, KO:K01734, KO:K03778
N676	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N856	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N665	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N775	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N857	5	KO:K00656, KO:K01069, KO:K01734, KO:K03778, KO:K10783
N802	5	KO:K00043, KO:K00627, KO:K00656, KO:K01442, KO:K01734
N801	5	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N774	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N847	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N648	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N862	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N852	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N841	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N860	5	KO:K00627, KO:K00656, KO:K01069, KO:K01734, KO:K03778
N864	5	KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N863	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N709	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N982	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N949	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N986	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N1047	4	KO:K00656, KO:K01659, KO:K01734, KO:K03778
N1086	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N1017	4	KO:K00656, KO:K01067, KO:K01442, KO:K01734
N899	4	KO:K00116, KO:K00656, KO:K01442, KO:K01734
N927	4	KO:K00656, KO:K01067, KO:K01442, KO:K01734
N969	4	KO:K00116, KO:K00656, KO:K01069, KO:K01734
N772	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N991	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N1079	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N1058	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N987	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N1063	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N1049	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N728	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N1009	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N1098	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N947	4	KO:K00627, KO:K00656, KO:K01442, KO:K01734
N883	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N934	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N905	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N963	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N1034	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N998	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N929	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N881	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N765	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N1081	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N1048	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N970	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N1024	4	KO:K00102, KO:K00656, KO:K01442, KO:K01734
N911	4	KO:K00656, KO:K01069, KO:K01734, KO:K10783
N1026	4	KO:K00627, KO:K00656, KO:K01442, KO:K01734
N942	4	KO:K00102, KO:K00656, KO:K01442, KO:K01734
N813	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N811	4	KO:K00116, KO:K00656, KO:K01069, KO:K01734
N810	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734

N808	4	KO:K00656, KO:K01069, KO:K01734, KO:K10783
N805	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N806	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N1088	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N781	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N776	4	KO:K00116, KO:K00656, KO:K01442, KO:K01734
N1020	3	KO:K00656, KO:K01442, KO:K01734
N930	3	KO:K00656, KO:K01442, KO:K01734
N996	3	KO:K00656, KO:K01442, KO:K01734
N1057	3	KO:K00656, KO:K01442, KO:K01734
N1089	3	KO:K00656, KO:K01659, KO:K01734
N1094	3	KO:K00627, KO:K00656, KO:K03778
N850	3	KO:K00656, KO:K01734, KO:K03778
N1090	2	KO:K00656, KO:K01442
N1092	2	KO:K01442, KO:K01734
N412	0	
N287	0	

Table 19.

KEGG Orthology Pathway (KO) hierarchical ontology for KOs identified in computationally determined networks characteristic of states of health and reported in Table 18.

KEGG number	KEGG annotation level 1	KEGG annotation level 2	KEGG annotation level 3	Full KEGG annotation level 4
KO:K01442	Metabolism	Lipid metabolism	00121 Secondary bile acid biosynthesis [PATH:ko00121]	E3.5.1.24; choloylglycine hydrolase [EC:3.5.1.24]
KO:K00004	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	BDH, butB; (R,R)-butanediol dehydrogenase / diacetyl reductase [EC:1.1.1.4 1.1.1.303]
KO:K00023	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	E1.1.1.36, phbB; acetoacetyl-CoA reductase [EC:1.1.1.36]
KO:K00043	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	E1.1.1.61; 4-hydroxybutyrate dehydrogenase [EC:1.1.1.61]
KO:K00101	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	E1.1.2.3, lldD; L-lactate dehydrogenase (cytochrome) [EC:1.1.2.3]
KO:K00102	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	E1.1.2.4, dld; D-lactate dehydrogenase (cytochrome) [EC:1.1.2.4]
KO:K00116	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	mqo; malate dehydrogenase (quinone) [EC:1.1.5.4]
KO:K00156	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	poxB; pyruvate dehydrogenase (quinone) [EC:1.2.5.1]
KO:K00158	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	E1.2.3.3, poxL; pyruvate oxidase [EC:1.2.3.3]
KO:K00163	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	aceE; pyruvate dehydrogenase E1 component [EC:1.2.4.1]
KO:K00627	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	DLAT, aceF, pdhC; pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) [EC:2.3.1.12]
KO:K00634	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	ptb; phosphate butyryltransferase [EC:2.3.1.19]
KO:K00656	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	E2.3.1.54, pflD; formate C-acetyltransferase [EC:2.3.1.54]
KO:K00929	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	E2.7.2.7, buk; butyrate kinase [EC:2.7.2.7]
KO:K00932	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E2.7.2.15, tdcD, pduW; propionate kinase [EC:2.7.2.15]
KO:K01067	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	E3.1.2.1, ACH1; acetyl-CoA hydrolase [EC:3.1.2.1]
KO:K01069	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	E3.1.2.6, gloB; hydroxyacylglutathione hydrolase [EC:3.1.2.6]

KEGG number	KEGG annotation level 1	KEGG annotation level 2	KEGG annotation level 3	Full KEGG annotation level 4
KO:K01505	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E3.5.99.7; 1-aminocyclopropane-1-carboxylate deaminase [EC:3.5.99.7]
KO:K01659	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E2.3.3.5, prpC; 2-methylcitrate synthase [EC:2.3.3.5]
KO:K01720	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E4.2.1.79, prpD; 2-methylcitrate dehydratase [EC:4.2.1.79]
KO:K01734	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	E4.2.3.3, mgsA; methylglyoxal synthase [EC:4.2.3.3]
KO:K01896	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	ACSM; medium-chain acyl-CoA synthetase [EC:6.2.1.2]
KO:K01905	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E6.2.1.13; acetyl-CoA synthetase (ADP-forming) [EC:6.2.1.13]
KO:K01907	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	AACS, acsA; acetoacetyl-CoA synthetase [EC:6.2.1.16]
KO:K01908	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E6.2.1.17, prpE; propionyl-CoA synthetase [EC:6.2.1.17]
KO:K03366	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	butA; (R,R)-butanediol dehydrogenase / diacetyl reductase [EC:1.1.1.4 1.1.1.303]
KO:K03416	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E2.1.3.1; methylmalonyl-CoA carboxyltransferase [EC:2.1.3.1]
KO:K03417	Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	E4.1.3.30, prpB; methylisocitrate lyase [EC:4.1.3.30]
KO:K03777	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	dld; D-lactate dehydrogenase [EC:1.1.1.28]
KO:K03778	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	ldhA; D-lactate dehydrogenase [EC:1.1.1.28]
KO:K03821	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	phbC, phaC; polyhydroxyalkanoate synthase [EC:2.3.1.-]
KO:K04020	Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	eutD; phosphotransacetylase
KO:K05973	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	E3.1.1.75, phaZ; poly(3-hydroxybutyrate) depolymerase [EC:3.1.1.75]
KO:K07246	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	ttuC, dmlA; tartrate dehydrogenase/decarboxylase / D-malate dehydrogenase [EC:1.1.1.93 4.1.1.73 1.1.1.83]
KO:K10783	Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	E1.3.1.44; trans-2-enoyl-CoA reductase (NAD+) [EC:1.3.1.44]
KO:K13923	Metabolism	Carbohydrate	00640 Propanoate metabolism	pduL; phosphotransacylase

KEGG number	KEGG annotation level 1	KEGG annotation level 2	KEGG annotation level 3	Full KEGG annotation level 4
		metabolism	[PATH:ko00640]	

Table 20.

Keystone OTUs inhibit *C. difficile* growth in a competitive in vitro simulation assay

OTU_1	OTU_1 is a Key stone	OTU_2	OTU_2 is a Key Stone	C. diff Inhibition Score
Bacteroides_caccae	1	Bacteroides_eggerthii		++++
Bacteroides_sp_D20	1	Bacteroides_eggerthii		
Clostridium_nexile	1	Bacteroides_eggerthii		
Coprococcus_catus	1	Bacteroides_eggerthii		+
Dorea_formicigenerans	1	Bacteroides_eggerthii		
Dorea_longicatena	1	Bacteroides_eggerthii		--
Faecalibacterium_prausnitzii	1	Bacteroides_eggerthii		
Odoribacter_splanchnicus	1	Bacteroides_eggerthii		
Parabacteroides_merdae	1	Bacteroides_eggerthii		
Roseburia_intestinalis	1	Bacteroides_eggerthii		
Ruminococcus_obeum	1	Bacteroides_eggerthii		++++
Bacteroides_caccae	1	Bifidobacterium_adolescentis		++++
Bacteroides_sp_D20	1	Bifidobacterium_adolescentis		++++
Clostridium_nexile	1	Bifidobacterium_adolescentis		++++
Coprococcus_catus	1	Bifidobacterium_adolescentis		
Dorea_formicigenerans	1	Bifidobacterium_adolescentis		++++
Dorea_longicatena	1	Bifidobacterium_adolescentis		++++
Faecalibacterium_prausnitzii	1	Bifidobacterium_adolescentis		+
Odoribacter_splanchnicus	1	Bifidobacterium_adolescentis		+++
Parabacteroides_merdae	1	Bifidobacterium_adolescentis		++++
Roseburia_intestinalis	1	Bifidobacterium_adolescentis		+++
Ruminococcus_obeum	1	Bifidobacterium_adolescentis		++++
Ruminococcus_torques	1	Bifidobacterium_adolescentis		++++
Bacteroides_caccae	1	Bifidobacterium_pseudocatenulatum		++++
Bacteroides_sp_D20	1	Bifidobacterium_pseudocatenulatum		--
Clostridium_nexile	1	Bifidobacterium_pseudocatenulatum		++++
Coprococcus_catus	1	Bifidobacterium_pseudocatenulatum		
Dorea_formicigenerans	1	Bifidobacterium_pseudocatenulatum		+++
Dorea_longicatena	1	Bifidobacterium_pseudocatenulatum		++++
Faecalibacterium_prausnitzii	1	Bifidobacterium_pseudocatenulatum		++
Odoribacter_splanchnicus	1	Bifidobacterium_pseudocatenulatum		+
Parabacteroides_merdae	1	Bifidobacterium_pseudocatenulatum		++++
Roseburia_intestinalis	1	Bifidobacterium_pseudocatenulatum		+++
Ruminococcus_obeum	1	Bifidobacterium_pseudocatenulatum		++++
Ruminococcus_torques	1	Bifidobacterium_pseudocatenulatum		++++
Bacteroides_caccae	1	Blautia_schinkii		
Bacteroides_sp_D20	1	Blautia_schinkii		--
Clostridium_nexile	1	Blautia_schinkii		--
Coprococcus_catus	1	Blautia_schinkii		---
Coprococcus_comes	1	Blautia_schinkii		+
Dorea_formicigenerans	1	Blautia_schinkii		
Dorea_longicatena	1	Blautia_schinkii		+++

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	C. diff inhibition score
Eubacterium_rectale	1	Blautia_schinkii		+
Faecalibacterium_prausnitzii	1	Blautia_schinkii		
Faecalibacterium_prausnitzii	1	Blautia_schinkii		
Odoribacter_splanchnicus	1	Blautia_schinkii		–
Odoribacter_splanchnicus	1	Blautia_schinkii		
Parabacteroides_merdae	1	Blautia_schinkii		
Roseburia_intestinalis	1	Blautia_schinkii		–
Ruminococcus_obeum	1	Blautia_schinkii		
Ruminococcus_torques	1	Blautia_schinkii		
Coprococcus_comes	1	Clostridium butyricum		++++
Coprococcus_comes	1	Clostridium orbiscindens		++++
Faecalibacterium_prausnitzii	1	Clostridium orbiscindens		
Coprococcus_comes	1	Clostridium_bolteae		++++
Coprococcus_comes	1	Clostridium_hylemonae		+
Clostridium_nexile	1	Clostridium_sp_HGF2		++++
Dorea_formicigenerans	1	Clostridium_sp_HGF2		
Clostridium_nexile	1	Erysipelotrichaceae_bacterium		
Coprococcus_catus	1	Erysipelotrichaceae_bacterium		
Dorea_formicigenerans	1	Erysipelotrichaceae_bacterium		----
Dorea_longicatena	1	Erysipelotrichaceae_bacterium		
Faecalibacterium_prausnitzii	1	Erysipelotrichaceae_bacterium		–
Odoribacter_splanchnicus	1	Erysipelotrichaceae_bacterium		
Roseburia_intestinalis	1	Erysipelotrichaceae_bacterium		–
Bacteroides_caccae	1	Lachnospiraceae_bacterium_5_1_57FAA		
Bacteroides_sp_D20	1	Lachnospiraceae_bacterium_5_1_57FAA		----
Clostridium_nexile	1	Lachnospiraceae_bacterium_5_1_57FAA		–
Coprococcus_catus	1	Lachnospiraceae_bacterium_5_1_57FAA		
Coprococcus_comes	1	Lachnospiraceae_bacterium_5_1_57FAA		
Coprococcus_comes	1	Lachnospiraceae_bacterium_5_1_57FAA		++++
Dorea_formicigenerans	1	Lachnospiraceae_bacterium_5_1_57FAA		—
Dorea_longicatena	1	Lachnospiraceae_bacterium_5_1_57FAA		++++
Eubacterium_rectale	1	Lachnospiraceae_bacterium_5_1_57FAA		
Faecalibacterium_prausnitzii	1	Lachnospiraceae_bacterium_5_1_57FAA		
Faecalibacterium_prausnitzii	1	Lachnospiraceae_bacterium_5_1_57FAA		
Faecalibacterium_prausnitzii	1	Lachnospiraceae_bacterium_5_1_57FAA		++++
Odoribacter_splanchnicus	1	Lachnospiraceae_bacterium_5_1_57FAA		----
Odoribacter_splanchnicus	1	Lachnospiraceae_bacterium_5_1_57FAA		
Parabacteroides_merdae	1	Lachnospiraceae_bacterium_5_1_57FAA		–
Roseburia_intestinalis	1	Lachnospiraceae_bacterium_5_1_57FAA		
Ruminococcus_obeum	1	Lachnospiraceae_bacterium_5_1_57FAA		
Ruminococcus_torques	1	Lachnospiraceae_bacterium_5_1_57FAA		
Bacteroides_eggerthii		Alistipes_shahii	1	—
Bacteroides_sp_1_1_6		Alistipes_shahii	1	++++
Bacteroides_sp_3_1_23		Alistipes_shahii	1	+

OTU_1	OTU_1 is a Key stone	OTU_2	OTU_2 is a key stone	4 diff Inhibition Score
Bifidobacterium_adolescentis		Alistipes_shahii	1	++++
Bifidobacterium_pseudocatenulatum		Alistipes_shahii	1	
Blautia_schinkii		Alistipes_shahii	1	
Clostridium_sp_HGF2		Alistipes_shahii	1	
Coprobacillus_sp_D7		Alistipes_shahii	1	++++
Enterococcus_faecalis		Alistipes_shahii	1	++++
Erysipelotrichaceae_bacterium		Alistipes_shahii	1	---
Lachnospiraceae_bacterium_5_1_57FAA		Alistipes_shahii	1	
Bacteroides_sp_1_1_6		Bacteroides_caccae	1	++++
Bacteroides_sp_3_1_23		Bacteroides_caccae	1	+++
Clostridium_sp_HGF2		Bacteroides_caccae	1	
Coprobacillus_sp_D7		Bacteroides_caccae	1	+++
Enterococcus_faecalis		Bacteroides_caccae	1	++++
Erysipelotrichaceae_bacterium		Bacteroides_caccae	1	++
Bacteroides_sp_1_1_6		Bacteroides_sp_D20	1	++++
Bacteroides_sp_3_1_23		Bacteroides_sp_D20	1	++++
Clostridium_sp_HGF2		Bacteroides_sp_D20	1	
Coprobacillus_sp_D7		Bacteroides_sp_D20	1	
Enterococcus_faecalis		Bacteroides_sp_D20	1	++++
Erysipelotrichaceae_bacterium		Bacteroides_sp_D20	1	
Bacteroides_sp_1_1_6		Clostridium_nexile	1	++++
Bacteroides_sp_3_1_23		Clostridium_nexile	1	++++
Coprobacillus_sp_D7		Clostridium_nexile	1	
Enterococcus_faecalis		Clostridium_nexile	1	++++
Bacteroides_sp_1_1_6		Coprococcus_catus	1	++++
Bacteroides_sp_3_1_23		Coprococcus_catus	1	
Clostridium_sp_HGF2		Coprococcus_catus	1	---
Coprobacillus_sp_D7		Coprococcus_catus	1	---
Enterococcus_faecalis		Coprococcus_catus	1	++++
Bacteroides_eggerthii		Coprococcus_comes	1	+++
Bacteroides_sp_1_1_6		Coprococcus_comes	1	+++
Bacteroides_sp_3_1_23		Coprococcus_comes	1	++++
Bifidobacterium_adolescentis		Coprococcus_comes	1	++++
Bifidobacterium_pseudocatenulatum		Coprococcus_comes	1	++++
Clostridium butyricum		Coprococcus_comes	1	++++
Clostridium_disporicum		Coprococcus_comes	1	++++
Clostridium_hylemonae		Coprococcus_comes	1	+
Clostridium_innocuum		Coprococcus_comes	1	++++
Clostridium_mayombei		Coprococcus_comes	1	++++
Clostridium_sp_HGF2		Coprococcus_comes	1	
Clostridium_tertium		Coprococcus_comes	1	++++
Coprobacillus_sp_D7		Coprococcus_comes	1	+++
Enterococcus_faecalis		Coprococcus_comes	1	++++
Erysipelotrichaceae_bacterium		Coprococcus_comes	1	–

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	C.diff inhibition score
Bacteroides_sp_1_1_6		Dorea_formicigenerans	1	+++
Bacteroides_sp_3_1_23		Dorea_formicigenerans	1	
Coprobacillus_sp_D7		Dorea_formicigenerans	1	–
Enterococcus_faecalis		Dorea_formicigenerans	1	++++
Bacteroides_sp_1_1_6		Dorea_longicatena	1	++++
Bacteroides_sp_3_1_23		Dorea_longicatena	1	+++
Clostridium_sp_HGF2		Dorea_longicatena	1	—
Coprobacillus_sp_D7		Dorea_longicatena	1	
Enterococcus_faecalis		Dorea_longicatena	1	++++
Bacteroides_eggerthii		Eubacterium_rectale	1	
Bacteroides_sp_1_1_6		Eubacterium_rectale	1	++++
Bacteroides_sp_3_1_23		Eubacterium_rectale	1	+++
Bifidobacterium_adolescentis		Eubacterium_rectale	1	++++
Bifidobacterium_pseudocatenulatum		Eubacterium_rectale	1	
Clostridium butyricum		Eubacterium_rectale	1	++++
Clostridium orbiscindens		Eubacterium_rectale	1	+
Clostridium_bolteae		Eubacterium_rectale	1	++
Clostridium_disporicum		Eubacterium_rectale	1	++++
Clostridium_hylemonae		Eubacterium_rectale	1	
Clostridium_innocuum		Eubacterium_rectale	1	++++
Clostridium_mayombei		Eubacterium_rectale	1	++++
Clostridium_sp_HGF2		Eubacterium_rectale	1	—
Clostridium_tertium		Eubacterium_rectale	1	++++
Coprobacillus_sp_D7		Eubacterium_rectale	1	+++
Enterococcus_faecalis		Eubacterium_rectale	1	++++
Erysipelotrichaceae_bacterium		Eubacterium_rectale	1	–
Lachnospiraceae_bacterium_5_1_57FAA		Eubacterium_rectale	1	+++
Bacteroides_eggerthii		Faecalibacterium_prausnitzii	1	–
Bacteroides_sp_1_1_6		Faecalibacterium_prausnitzii	1	++++
Bacteroides_sp_1_1_6		Faecalibacterium_prausnitzii	1	+++
Bacteroides_sp_3_1_23		Faecalibacterium_prausnitzii	1	++
Bacteroides_sp_3_1_23		Faecalibacterium_prausnitzii	1	
Bifidobacterium_adolescentis		Faecalibacterium_prausnitzii	1	+
Bifidobacterium_pseudocatenulatum		Faecalibacterium_prausnitzii	1	
Clostridium butyricum		Faecalibacterium_prausnitzii	1	++++
Clostridium orbiscindens		Faecalibacterium_prausnitzii	1	
Clostridium_bolteae		Faecalibacterium_prausnitzii	1	++
Clostridium_disporicum		Faecalibacterium_prausnitzii	1	
Clostridium_hylemonae		Faecalibacterium_prausnitzii	1	
Clostridium_innocuum		Faecalibacterium_prausnitzii	1	++++
Clostridium_mayombei		Faecalibacterium_prausnitzii	1	++++
Clostridium_sp_HGF2		Faecalibacterium_prausnitzii	1	++
Clostridium_sp_HGF2		Faecalibacterium_prausnitzii	1	—
Clostridium_tertium		Faecalibacterium_prausnitzii	1	++++

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	C.diff inhibition score
Coprobacillus_sp_D7		Faecalibacterium_prausnitzii	1	------
Coprobacillus_sp_D7		Faecalibacterium_prausnitzii	1	
Enterococcus_faecalis		Faecalibacterium_prausnitzii	1	++++
Enterococcus_faecalis		Faecalibacterium_prausnitzii	1	++++
Erysipelotrichaceae_bacterium		Faecalibacterium_prausnitzii	1	--
Lachnospiraceae_bacterium_5_1_57FAA		Faecalibacterium_prausnitzii	1	++++
Bacteroides_eggerthii		Odoribacter_splanchnicus	1	-
Bacteroides_sp_1_1_6		Odoribacter_splanchnicus	1	++++
Bacteroides_sp_1_1_6		Odoribacter_splanchnicus	1	+
Bacteroides_sp_3_1_23		Odoribacter_splanchnicus	1	+
Bacteroides_sp_3_1_23		Odoribacter_splanchnicus	1	
Bifidobacterium_adolescentis		Odoribacter_splanchnicus	1	++++
Bifidobacterium_pseudocatenulatum		Odoribacter_splanchnicus	1	+++
Clostridium_sp_HGF2		Odoribacter_splanchnicus	1	
Clostridium_sp_HGF2		Odoribacter_splanchnicus	1	---
Coprobacillus_sp_D7		Odoribacter_splanchnicus	1	-
Coprobacillus_sp_D7		Odoribacter_splanchnicus	1	+++
Enterococcus_faecalis		Odoribacter_splanchnicus	1	++++
Enterococcus_faecalis		Odoribacter_splanchnicus	1	++++
Erysipelotrichaceae_bacterium		Odoribacter_splanchnicus	1	--
Bacteroides_sp_1_1_6		Parabacteroides_merdae	1	++++
Bacteroides_sp_3_1_23		Parabacteroides_merdae	1	+++
Clostridium_sp_HGF2		Parabacteroides_merdae	1	+++
Coprobacillus_sp_D7		Parabacteroides_merdae	1	-
Enterococcus_faecalis		Parabacteroides_merdae	1	++++
Erysipelotrichaceae_bacterium		Parabacteroides_merdae	1	
Bacteroides_sp_1_1_6		Roseburia_intestinalis	1	++++
Bacteroides_sp_3_1_23		Roseburia_intestinalis	1	+
Clostridium_sp_HGF2		Roseburia_intestinalis	1	---
Coprobacillus_sp_D7		Roseburia_intestinalis	1	-
Enterococcus_faecalis		Roseburia_intestinalis	1	++++
Clostridium butyricum		Ruminococcus_bromii	1	++++
Clostridium orbiscindens		Ruminococcus_bromii	1	
Clostridium_bolteae		Ruminococcus_bromii	1	+++
Clostridium_disporicum		Ruminococcus_bromii	1	
Clostridium_hylemonae		Ruminococcus_bromii	1	
Clostridium_innocuum		Ruminococcus_bromii	1	++++
Clostridium_mayombei		Ruminococcus_bromii	1	++++
Clostridium_tertium		Ruminococcus_bromii	1	++++
Lachnospiraceae_bacterium_5_1_57FAA		Ruminococcus_bromii	1	++++
Bacteroides_sp_1_1_6		Ruminococcus_obeum	1	+++
Bacteroides_sp_3_1_23		Ruminococcus_obeum	1	+++
Clostridium_sp_HGF2		Ruminococcus_obeum	1	--
Coprobacillus_sp_D7		Ruminococcus_obeum	1	+

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	t_diff inhibition score
Enterococcus_faecalis		Ruminococcus_obeum	1	++++
Erysipelotrichaceae_bacterium		Ruminococcus_obeum	1	
Bacteroides_eggerthii		Ruminococcus_torques	1	++++
Bacteroides_sp_1_1_6		Ruminococcus_torques	1	++++
Bacteroides_sp_3_1_23		Ruminococcus_torques	1	++++
Clostridium_sp_HGF2		Ruminococcus_torques	1	
Coprobacillus_sp_D7		Ruminococcus_torques	1	++++
Enterococcus_faecalis		Ruminococcus_torques	1	++++
Erysipelotrichaceae_bacterium		Ruminococcus_torques	1	+
Alistipes_shahii	1	Alistipes_shahii	1	
Bacteroides_caccae	1	Alistipes_shahii	1	
Bacteroides_sp_D20	1	Alistipes_shahii	1	–
Clostridium_nexile	1	Alistipes_shahii	1	
Coprococcus_catus	1	Alistipes_shahii	1	–
Coprococcus_comes	1	Alistipes_shahii	1	
Dorea_formicigenerans	1	Alistipes_shahii	1	– – – –
Dorea_longicatena	1	Alistipes_shahii	1	++++
Eubacterium_rectale	1	Alistipes_shahii	1	
Faecalibacterium_prausnitzii	1	Alistipes_shahii	1	
Faecalibacterium_prausnitzii	1	Alistipes_shahii	1	+
Odoribacter_splanchnicus	1	Alistipes_shahii	1	
Odoribacter_splanchnicus	1	Alistipes_shahii	1	
Parabacteroides_merdae	1	Alistipes_shahii	1	
Roseburia_intestinalis	1	Alistipes_shahii	1	–
Ruminococcus_obeum	1	Alistipes_shahii	1	
Ruminococcus_torques	1	Alistipes_shahii	1	
Bacteroides_caccae	1	Bacteroides_caccae	1	++++
Bacteroides_sp_D20	1	Bacteroides_caccae	1	+++
Clostridium_nexile	1	Bacteroides_caccae	1	
Coprococcus_catus	1	Bacteroides_caccae	1	++++
Dorea_formicigenerans	1	Bacteroides_caccae	1	+++
Dorea_longicatena	1	Bacteroides_caccae	1	
Faecalibacterium_prausnitzii	1	Bacteroides_caccae	1	–
Odoribacter_splanchnicus	1	Bacteroides_caccae	1	
Parabacteroides_merdae	1	Bacteroides_caccae	1	+
Roseburia_intestinalis	1	Bacteroides_caccae	1	+
Ruminococcus_obeum	1	Bacteroides_caccae	1	++++
Bacteroides_sp_D20	1	Bacteroides_sp_D20	1	
Clostridium_nexile	1	Bacteroides_sp_D20	1	–
Coprococcus_catus	1	Bacteroides_sp_D20	1	
Dorea_formicigenerans	1	Bacteroides_sp_D20	1	–
Dorea_longicatena	1	Bacteroides_sp_D20	1	
Faecalibacterium_prausnitzii	1	Bacteroides_sp_D20	1	
Odoribacter_splanchnicus	1	Bacteroides_sp_D20	1	

OTU_1	OTU_1 is a Key stone	OTU_2	OTU_2 is a key Stone	C.diff Inhibition Score
Roseburia_intestinalis	1	Bacteroides_sp_D20	1	–
Clostridium_nexile	1	Clostridium_nexile	1	++
Dorea_formicigenerans	1	Clostridium_nexile	1	
Clostridium_nexile	1	Coprococcus_catus	1	
Coprococcus_catus	1	Coprococcus_catus	1	
Dorea_formicigenerans	1	Coprococcus_catus	1	
Dorea_longicatena	1	Coprococcus_catus	1	
Faecalibacterium_prausnitzii	1	Coprococcus_catus	1	
Odoribacter_splanchnicus	1	Coprococcus_catus	1	
Roseburia_intestinalis	1	Coprococcus_catus	1	
Bacteroides_caccae	1	Coprococcus_comes	1	+++
Bacteroides_sp_D20	1	Coprococcus_comes	1	
Clostridium_nexile	1	Coprococcus_comes	1	
Coprococcus_catus	1	Coprococcus_comes	1	—
Coprococcus_comes	1	Coprococcus_comes	1	
Coprococcus_comes	1	Coprococcus_comes	1	++
Dorea_formicigenerans	1	Coprococcus_comes	1	
Dorea_longicatena	1	Coprococcus_comes	1	
Faecalibacterium_prausnitzii	1	Coprococcus_comes	1	
Odoribacter_splanchnicus	1	Coprococcus_comes	1	
Parabacteroides_merdae	1	Coprococcus_comes	1	–
Roseburia_intestinalis	1	Coprococcus_comes	1	–
Ruminococcus_obeum	1	Coprococcus_comes	1	++++
Ruminococcus_torques	1	Coprococcus_comes	1	++++
Dorea_formicigenerans	1	Dorea_formicigenerans	1	—
Clostridium_nexile	1	Dorea_longicatena	1	
Dorea_formicigenerans	1	Dorea_longicatena	1	++
Dorea_longicatena	1	Dorea_longicatena	1	–
Faecalibacterium_prausnitzii	1	Dorea_longicatena	1	–
Odoribacter_splanchnicus	1	Dorea_longicatena	1	
Bacteroides_caccae	1	Eubacterium_rectale	1	
Bacteroides_sp_D20	1	Eubacterium_rectale	1	—
Clostridium_nexile	1	Eubacterium_rectale	1	–
Coprococcus_catus	1	Eubacterium_rectale	1	—
Coprococcus_comes	1	Eubacterium_rectale	1	+
Coprococcus_comes	1	Eubacterium_rectale	1	++++
Dorea_formicigenerans	1	Eubacterium_rectale	1	–
Dorea_longicatena	1	Eubacterium_rectale	1	++++
Eubacterium_rectale	1	Eubacterium_rectale	1	+++
Eubacterium_rectale	1	Eubacterium_rectale	1	
Faecalibacterium_prausnitzii	1	Eubacterium_rectale	1	—
Faecalibacterium_prausnitzii	1	Eubacterium_rectale	1	
Odoribacter_splanchnicus	1	Eubacterium_rectale	1	–
Parabacteroides_merdae	1	Eubacterium_rectale	1	–

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	4 diff inhibition score
Roseburia_intestinalis	1	Eubacterium_rectale	1	------
Ruminococcus_bromii	1	Eubacterium_rectale	1	+
Ruminococcus_obeum	1	Eubacterium_rectale	1	++
Ruminococcus_torques	1	Eubacterium_rectale	1	+
Bacteroides_caccae	1	Faecalibacterium_prausnitzii	1	
Bacteroides_sp_D20	1	Faecalibacterium_prausnitzii	1	—
Clostridium_nexile	1	Faecalibacterium_prausnitzii	1	
Clostridium_nexile	1	Faecalibacterium_prausnitzii	1	–
Coprococcus_catus	1	Faecalibacterium_prausnitzii	1	------
Coprococcus_comes	1	Faecalibacterium_prausnitzii	1	
Coprococcus_comes	1	Faecalibacterium_prausnitzii	1	+++
Dorea_formicigenerans	1	Faecalibacterium_prausnitzii	1	
Dorea_formicigenerans	1	Faecalibacterium_prausnitzii	1	---
Dorea_longicatena	1	Faecalibacterium_prausnitzii	1	+++
Eubacterium_rectale	1	Faecalibacterium_prausnitzii	1	+
Faecalibacterium_prausnitzii	1	Faecalibacterium_prausnitzii	1	+
Faecalibacterium_prausnitzii	1	Faecalibacterium_prausnitzii	1	
Faecalibacterium_prausnitzii	1	Faecalibacterium_prausnitzii	1	+
Faecalibacterium_prausnitzii	1	Faecalibacterium_prausnitzii	1	
Odoribacter_splanchnicus	1	Faecalibacterium_prausnitzii	1	—
Parabacteroides_merdae	1	Faecalibacterium_prausnitzii	1	–
Roseburia_intestinalis	1	Faecalibacterium_prausnitzii	1	
Ruminococcus_obeum	1	Faecalibacterium_prausnitzii	1	
Ruminococcus_torques	1	Faecalibacterium_prausnitzii	1	
Bacteroides_caccae	1	Odoribacter_splanchnicus	1	
Bacteroides_sp_D20	1	Odoribacter_splanchnicus	1	---
Clostridium_nexile	1	Odoribacter_splanchnicus	1	
Clostridium_nexile	1	Odoribacter_splanchnicus	1	------
Coprococcus_catus	1	Odoribacter_splanchnicus	1	—
Coprococcus_comes	1	Odoribacter_splanchnicus	1	
Dorea_formicigenerans	1	Odoribacter_splanchnicus	1	
Dorea_formicigenerans	1	Odoribacter_splanchnicus	1	–
Dorea_longicatena	1	Odoribacter_splanchnicus	1	++++
Eubacterium_rectale	1	Odoribacter_splanchnicus	1	+
Faecalibacterium_prausnitzii	1	Odoribacter_splanchnicus	1	
Faecalibacterium_prausnitzii	1	Odoribacter_splanchnicus	1	–
Faecalibacterium_prausnitzii	1	Odoribacter_splanchnicus	1	+
Odoribacter_splanchnicus	1	Odoribacter_splanchnicus	1	
Odoribacter_splanchnicus	1	Odoribacter_splanchnicus	1	---
Odoribacter_splanchnicus	1	Odoribacter_splanchnicus	1	+
Parabacteroides_merdae	1	Odoribacter_splanchnicus	1	
Roseburia_intestinalis	1	Odoribacter_splanchnicus	1	
Ruminococcus_obeum	1	Odoribacter_splanchnicus	1	+
Ruminococcus_torques	1	Odoribacter_splanchnicus	1	

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	C.diff inhibition score
Bacteroides_sp_D20	1	Parabacteroides_merdae	1	
Clostridium_nexile	1	Parabacteroides_merdae	1	++
Coprococcus_catus	1	Parabacteroides_merdae	1	+++
Dorea_formicigenerans	1	Parabacteroides_merdae	1	
Dorea_longicatena	1	Parabacteroides_merdae	1	
Faecalibacterium_prausnitzii	1	Parabacteroides_merdae	1	+
Odoribacter_splanchnicus	1	Parabacteroides_merdae	1	
Parabacteroides_merdae	1	Parabacteroides_merdae	1	+++
Roseburia_intestinalis	1	Parabacteroides_merdae	1	
Clostridium_nexile	1	Roseburia_intestinalis	1	--
Dorea_formicigenerans	1	Roseburia_intestinalis	1	
Dorea_longicatena	1	Roseburia_intestinalis	1	-
Faecalibacterium_prausnitzii	1	Roseburia_intestinalis	1	
Odoribacter_splanchnicus	1	Roseburia_intestinalis	1	-
Roseburia_intestinalis	1	Roseburia_intestinalis	1	
Coprococcus_comes	1	Ruminococcus_bromii	1	++++
Eubacterium_rectale	1	Ruminococcus_bromii	1	+
Faecalibacterium_prausnitzii	1	Ruminococcus_bromii	1	
Ruminococcus_bromii	1	Ruminococcus_bromii	1	--
Bacteroides_sp_D20	1	Ruminococcus_obeum	1	
Clostridium_nexile	1	Ruminococcus_obeum	1	-
Coprococcus_catus	1	Ruminococcus_obeum	1	
Dorea_formicigenerans	1	Ruminococcus_obeum	1	++++
Dorea_longicatena	1	Ruminococcus_obeum	1	-
Faecalibacterium_prausnitzii	1	Ruminococcus_obeum	1	
Odoribacter_splanchnicus	1	Ruminococcus_obeum	1	--
Parabacteroides_merdae	1	Ruminococcus_obeum	1	
Roseburia_intestinalis	1	Ruminococcus_obeum	1	
Ruminococcus_obeum	1	Ruminococcus_obeum	1	++++
Bacteroides_caccae	1	Ruminococcus_torques	1	++++
Bacteroides_sp_D20	1	Ruminococcus_torques	1	++
Clostridium_nexile	1	Ruminococcus_torques	1	+
Coprococcus_catus	1	Ruminococcus_torques	1	+
Dorea_formicigenerans	1	Ruminococcus_torques	1	++++
Dorea_longicatena	1	Ruminococcus_torques	1	
Faecalibacterium_prausnitzii	1	Ruminococcus_torques	1	
Odoribacter_splanchnicus	1	Ruminococcus_torques	1	
Parabacteroides_merdae	1	Ruminococcus_torques	1	+
Roseburia_intestinalis	1	Ruminococcus_torques	1	+
Ruminococcus_obeum	1	Ruminococcus_torques	1	++++
Ruminococcus_torques	1	Ruminococcus_torques	1	++++
Bacteroides_ovatus		Alistipes_shahii	1	
Bacteroides_vulgatus		Alistipes_shahii	1	+++
Bacteroides_vulgatus		Alistipes_shahii	1	+

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	1 diff inhibition score
Blautia_producta		Alistipes_shahii	1	++++
Clostridium_hathewayi		Alistipes_shahii	1	++++
Clostridium_symbiosum		Alistipes_shahii	1	
Collinsella_aerofaciens		Alistipes_shahii	1	++++
Escherichia_coli		Alistipes_shahii	1	++++
Escherichia_coli		Alistipes_shahii	1	++++
Eubacterium_eligens		Alistipes_shahii	1	—
Streptococcus_thermophilus		Alistipes_shahii	1	
Bacteroides_ovatus		Bacteroides_caccae	1	
Bacteroides_vulgatus		Bacteroides_caccae	1	++++
Bacteroides_vulgatus		Bacteroides_caccae	1	+
Blautia_producta		Bacteroides_caccae	1	++++
Collinsella_aerofaciens		Bacteroides_caccae	1	++++
Escherichia_coli		Bacteroides_caccae	1	++++
Escherichia_coli		Bacteroides_caccae	1	++++
Eubacterium_eligens		Bacteroides_caccae	1	++
Streptococcus_thermophilus		Bacteroides_caccae	1	++
Bacteroides_vulgatus		Bacteroides_sp_D20	1	+
Blautia_producta		Bacteroides_sp_D20	1	++++
Escherichia_coli		Bacteroides_sp_D20	1	++++
Eubacterium_eligens		Bacteroides_sp_D20	1	–
Streptococcus_thermophilus		Bacteroides_sp_D20	1	+
Bacteroides_vulgatus		Clostridium_nexile	1	++++
Blautia_producta		Clostridium_nexile	1	++++
Escherichia_coli		Clostridium_nexile	1	++++
Eubacterium_eligens		Clostridium_nexile	1	+
Streptococcus_thermophilus		Clostridium_nexile	1	+
Bacteroides_vulgatus		Coprococcus_catus	1	+
Blautia_producta		Coprococcus_catus	1	++++
Escherichia_coli		Coprococcus_catus	1	++++
Eubacterium_eligens		Coprococcus_catus	1	
Streptococcus_thermophilus		Coprococcus_catus	1	
Bacteroides_ovatus		Coprococcus_comes	1	
Bacteroides_vulgatus		Coprococcus_comes	1	++++
Bacteroides_vulgatus		Coprococcus_comes	1	
Blautia_producta		Coprococcus_comes	1	++++
Clostridium_hathewayi		Coprococcus_comes	1	++++
Collinsella_aerofaciens		Coprococcus_comes	1	++++
Collinsella_aerofaciens		Coprococcus_comes	1	+++
Escherichia_coli		Coprococcus_comes	1	++++
Escherichia_coli		Coprococcus_comes	1	++++
Eubacterium_eligens		Coprococcus_comes	1	++
Streptococcus_thermophilus		Coprococcus_comes	1	++
Bacteroides_vulgatus		Dorea_formicigenerans	1	++

OTU_1	OTU_1 is a Key stone	OTU_2	OTU_2 is a key stone	1 diff Inhibition Score
Escherichia_coli		Dorea_formicigenerans	1	++
Streptococcus_thermophilus		Dorea_formicigenerans	1	
Bacteroides_vulgatus		Dorea_longicatena	1	++++
Blautia_producta		Dorea_longicatena	1	++++
Escherichia_coli		Dorea_longicatena	1	++++
Eubacterium_eligens		Dorea_longicatena	1	++
Streptococcus_thermophilus		Dorea_longicatena	1	+
Bacteroides_ovatus		Eubacterium_rectale	1	
Bacteroides_vulgatus		Eubacterium_rectale	1	++++
Bacteroides_vulgatus		Eubacterium_rectale	1	
Blautia_producta		Eubacterium_rectale	1	++++
Blautia_producta		Eubacterium_rectale	1	++++
Clostridium_hathewayi		Eubacterium_rectale	1	++++
Clostridium_symbiosum		Eubacterium_rectale	1	++
Clostridium_symbiosum		Eubacterium_rectale	1	+
Collinsella_aerofaciens		Eubacterium_rectale	1	++++
Collinsella_aerofaciens		Eubacterium_rectale	1	++++
Escherichia_coli		Eubacterium_rectale	1	++++
Escherichia_coli		Eubacterium_rectale	1	++++
Eubacterium_eligens		Eubacterium_rectale	1	
Ruminococcus_gnavus		Eubacterium_rectale	1	++++
Streptococcus_thermophilus		Eubacterium_rectale	1	
Bacteroides_ovatus		Faecalibacterium_prausnitzii	1	–
Bacteroides_vulgatus		Faecalibacterium_prausnitzii	1	++++
Bacteroides_vulgatus		Faecalibacterium_prausnitzii	1	+++
Bacteroides_vulgatus		Faecalibacterium_prausnitzii	1	---
Blautia_producta		Faecalibacterium_prausnitzii	1	++++
Blautia_producta		Faecalibacterium_prausnitzii	1	++++
Clostridium_hathewayi		Faecalibacterium_prausnitzii	1	+++
Clostridium_symbiosum		Faecalibacterium_prausnitzii	1	+++
Clostridium_symbiosum		Faecalibacterium_prausnitzii	1	++++
Collinsella_aerofaciens		Faecalibacterium_prausnitzii	1	++++
Collinsella_aerofaciens		Faecalibacterium_prausnitzii	1	++++
Escherichia_coli		Faecalibacterium_prausnitzii	1	++++
Escherichia_coli		Faecalibacterium_prausnitzii	1	++++
Escherichia_coli		Faecalibacterium_prausnitzii	1	++
Eubacterium_eligens		Faecalibacterium_prausnitzii	1	
Eubacterium_eligens		Faecalibacterium_prausnitzii	1	
Streptococcus_thermophilus		Faecalibacterium_prausnitzii	1	
Streptococcus_thermophilus		Faecalibacterium_prausnitzii	1	
Bacteroides_ovatus		Odoribacter_splanchnicus	1	--
Bacteroides_vulgatus		Odoribacter_splanchnicus	1	+++
Bacteroides_vulgatus		Odoribacter_splanchnicus	1	+++
Bacteroides_vulgatus		Odoribacter_splanchnicus	1	–

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	C. diff inhibition score
Blautia_producta		Odoribacter_splanchnicus	1	++++
Blautia_producta		Odoribacter_splanchnicus	1	++++
Clostridium_hathewayi		Odoribacter_splanchnicus	1	++++
Clostridium_symbiosum		Odoribacter_splanchnicus	1	++
Collinsella_aerofaciens		Odoribacter_splanchnicus	1	++++
Escherichia_coli		Odoribacter_splanchnicus	1	++++
Escherichia_coli		Odoribacter_splanchnicus	1	++++
Escherichia_coli		Odoribacter_splanchnicus	1	++++
Eubacterium_eligens		Odoribacter_splanchnicus	1	
Eubacterium_eligens		Odoribacter_splanchnicus	1	
Streptococcus_thermophilus		Odoribacter_splanchnicus	1	
Streptococcus_thermophilus		Odoribacter_splanchnicus	1	+
Bacteroides_ovatus		Parabacteroides_merdae	1	
Bacteroides_vulgatus		Parabacteroides_merdae	1	++++
Blautia_producta		Parabacteroides_merdae	1	++++
Escherichia_coli		Parabacteroides_merdae	1	++++
Eubacterium_eligens		Parabacteroides_merdae	1	
Streptococcus_thermophilus		Parabacteroides_merdae	1	
Bacteroides_vulgatus		Roseburia_intestinalis	1	+
Blautia_producta		Roseburia_intestinalis	1	++++
Escherichia_coli		Roseburia_intestinalis	1	++++
Eubacterium_eligens		Roseburia_intestinalis	1	
Streptococcus_thermophilus		Roseburia_intestinalis	1	
Blautia_producta		Ruminococcus_bromii	1	++++
Clostridium_symbiosum		Ruminococcus_bromii	1	++++
Collinsella_aerofaciens		Ruminococcus_bromii	1	++++
Ruminococcus_gnavus		Ruminococcus_bromii	1	++++
Bacteroides_ovatus		Ruminococcus_obeum	1	
Bacteroides_vulgatus		Ruminococcus_obeum	1	++++
Bacteroides_vulgatus		Ruminococcus_obeum	1	
Blautia_producta		Ruminococcus_obeum	1	++++
Collinsella_aerofaciens		Ruminococcus_obeum	1	++++
Escherichia_coli		Ruminococcus_obeum	1	+++
Escherichia_coli		Ruminococcus_obeum	1	++++
Eubacterium_eligens		Ruminococcus_obeum	1	+
Streptococcus_thermophilus		Ruminococcus_obeum	1	+++
Bacteroides_ovatus		Ruminococcus_torques	1	++++
Bacteroides_vulgatus		Ruminococcus_torques	1	++++
Bacteroides_vulgatus		Ruminococcus_torques	1	++++
Blautia_producta		Ruminococcus_torques	1	++++
Collinsella_aerofaciens		Ruminococcus_torques	1	++++
Escherichia_coli		Ruminococcus_torques	1	++++
Escherichia_coli		Ruminococcus_torques	1	++++
Eubacterium_eligens		Ruminococcus_torques	1	++

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	t-diff inhibition score
Streptococcus_thermophilus		Ruminococcus_torques	1	+
Bacteroides_sp_D20	1	Bacteroides_ovatus		–
Clostridium_nexile	1	Bacteroides_ovatus		
Coprococcus_catus	1	Bacteroides_ovatus		
Dorea_formicigenerans	1	Bacteroides_ovatus		
Dorea_longicatena	1	Bacteroides_ovatus		–
Faecalibacterium_prausnitzii	1	Bacteroides_ovatus		
Odoribacter_splanchnicus	1	Bacteroides_ovatus		
Roseburia_intestinalis	1	Bacteroides_ovatus		
Bacteroides_sp_D20	1	Bacteroides_vulgatus		
Clostridium_nexile	1	Bacteroides_vulgatus		
Coprococcus_catus	1	Bacteroides_vulgatus		++
Dorea_formicigenerans	1	Bacteroides_vulgatus		
Dorea_longicatena	1	Bacteroides_vulgatus		
Faecalibacterium_prausnitzii	1	Bacteroides_vulgatus		
Odoribacter_splanchnicus	1	Bacteroides_vulgatus		
Parabacteroides_merdae	1	Bacteroides_vulgatus		+
Roseburia_intestinalis	1	Bacteroides_vulgatus		
Alistipes_shahii	1	Blautia_producta		
Bacteroides_caccae	1	Blautia_producta		+
Bacteroides_sp_D20	1	Blautia_producta		
Clostridium_nexile	1	Blautia_producta		–
Coprococcus_catus	1	Blautia_producta		----
Coprococcus_comes	1	Blautia_producta		
Coprococcus_comes	1	Blautia_producta		++++
Dorea_formicigenerans	1	Blautia_producta		++++
Dorea_formicigenerans	1	Blautia_producta		----
Dorea_longicatena	1	Blautia_producta		+++
Eubacterium_rectale	1	Blautia_producta		+
Faecalibacterium_prausnitzii	1	Blautia_producta		
Faecalibacterium_prausnitzii	1	Blautia_producta		+
Faecalibacterium_prausnitzii	1	Blautia_producta		++++
Odoribacter_splanchnicus	1	Blautia_producta		–
Odoribacter_splanchnicus	1	Blautia_producta		+
Parabacteroides_merdae	1	Blautia_producta		+++
Roseburia_intestinalis	1	Blautia_producta		----
Ruminococcus_obeum	1	Blautia_producta		
Ruminococcus_torques	1	Blautia_producta		
Bacteroides_caccae	1	Clostridium_hathewayi		++++
Bacteroides_sp_D20	1	Clostridium_hathewayi		++++
Clostridium_nexile	1	Clostridium_hathewayi		
Coprococcus_catus	1	Clostridium_hathewayi		+++
Dorea_formicigenerans	1	Clostridium_hathewayi		++++
Dorea_longicatena	1	Clostridium_hathewayi		+

OTU_1	OTU_1 is a key stone	OTU_2	OTU_2 is a key stone	C.diff inhibition score
Faecalibacterium_prausnitzii	1	Clostridium_hathewayi		
Odoribacter_splanchnicus	1	Clostridium_hathewayi		
Parabacteroides_merdae	1	Clostridium_hathewayi		+
Roseburia_intestinalis	1	Clostridium_hathewayi		+++
Ruminococcus_obeum	1	Clostridium_hathewayi		++++
Ruminococcus_torques	1	Clostridium_hathewayi		++++
Bacteroides_caccae	1	Clostridium_symbiosum		+++
Bacteroides_sp_D20	1	Clostridium_symbiosum		
Clostridium_nexile	1	Clostridium_symbiosum		+
Coprococcus_catus	1	Clostridium_symbiosum		--
Coprococcus_comes	1	Clostridium_symbiosum		
Coprococcus_comes	1	Clostridium_symbiosum		++++
Dorea_formicigenerans	1	Clostridium_symbiosum		
Dorea_longicatena	1	Clostridium_symbiosum		++++
Faecalibacterium_prausnitzii	1	Clostridium_symbiosum		
Odoribacter_splanchnicus	1	Clostridium_symbiosum		
Parabacteroides_merdae	1	Clostridium_symbiosum		--
Roseburia_intestinalis	1	Clostridium_symbiosum		----
Ruminococcus_obeum	1	Clostridium_symbiosum		++++
Ruminococcus_torques	1	Clostridium_symbiosum		++
Bacteroides_sp_D20	1	Collinsella_aerofaciens		++++
Clostridium_nexile	1	Collinsella_aerofaciens		+
Coprococcus_catus	1	Collinsella_aerofaciens		++++
Dorea_formicigenerans	1	Collinsella_aerofaciens		++
Dorea_longicatena	1	Collinsella_aerofaciens		++++
Faecalibacterium_prausnitzii	1	Collinsella_aerofaciens		+++
Odoribacter_splanchnicus	1	Collinsella_aerofaciens		+++
Parabacteroides_merdae	1	Collinsella_aerofaciens		++++
Roseburia_intestinalis	1	Collinsella_aerofaciens		++
Bacteroides_sp_D20	1	Escherichia_coli		++++
Clostridium_nexile	1	Escherichia_coli		++++
Coprococcus_catus	1	Escherichia_coli		++++
Dorea_formicigenerans	1	Escherichia_coli		++++
Dorea_longicatena	1	Escherichia_coli		++++
Faecalibacterium_prausnitzii	1	Escherichia_coli		+++
Odoribacter_splanchnicus	1	Escherichia_coli		+++
Parabacteroides_merdae	1	Escherichia_coli		++++
Roseburia_intestinalis	1	Escherichia_coli		+++
Dorea_formicigenerans	1	Eubacterium_eligens		--
Coprococcus_comes	1	Ruminococcus_gnavus		++++
Faecalibacterium_prausnitzii	1	Ruminococcus_gnavus		++++
Ruminococcus_bromii	1	Ruminococcus_gnavus		++++

Table 21

Functional Network Ecologies comprised of two or more OTUs that are observed in the ethanol-treated spore preparation or the combined engrafted and augmented ecologies of at least one patient post-treatment with the bacterial composition. Network Ecology IDs with a ".s" indicates that the network is a subset of the computationally determined networks reported in Table 8 with the same Network Ecology ID.

Net ID	# Score	KEGG Orthology Pathways
N968.S	16	KO:K00101, KO:K00116, KO:K00156, KO:K00163, KO:K00627, KO:K00656, KO:K00932, KO:K01069, KO:K01659, KO:K01720, KO:K01734, KO:K01908, KO:K03417, KO:K03777, KO:K03778, KO:K04020
N282.S	12	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N390.S	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N515.S	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N516.S	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N586	11	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N590.S	11	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N666.S	11	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N271.S	10	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N338.S	10	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N368.S	10	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N381.S	10	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N382.S	10	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K01905, KO:K03417, KO:K07246
N519.S	10	KO:K00004, KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366, KO:K03778
N535.S	10	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N597.S	10	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N680.S	10	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K01905, KO:K03778
N822.S	10	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N1008	9	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01659, KO:K01734, KO:K03778, KO:K04020
N329.S	9	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N332.S	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N346.S	9	KO:K00116, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N353.S	9	KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N361.S	9	KO:K00116, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N375.S	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N377.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N380.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N387.S	9	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N396.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N397.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N415.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N419.S	9	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N431.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N434.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N440.S	9	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N447.S	9	KO:K00634, KO:K00656, KO:K00929, KO:K01067, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N458.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N459.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N481.S	9	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N526	9	KO:K00004, KO:K00116, KO:K00163, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N530.S	9	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N534.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N591.S	9	KO:K00004, KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N682.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N816.S	9	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734,

		KO:K03778
N891.S	9	KO:K00102, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K01905, KO:K03778
N1091	8	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734
N1093	8	KO:K00156, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N262.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N279.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N284.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N288.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N290.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N301.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N302.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N310.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N312.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N323.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N325.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N331.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N336.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N339.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N340.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N341.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N343.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N344.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N345.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N352.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N355.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N356.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N357.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N358.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N369.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N370.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N372.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N373.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N374.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N376.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N386.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N389.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N394.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N400.S	8	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K07246
N405.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N408.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N422.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N423.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N429.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N430.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N432.S	8	KO:K00627, KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N437.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N450.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N477.S	8	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N478.S	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N482.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N490.S	8	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N493.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N509.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N543.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N547.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N582.S	8	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N584.S	8	KO:K00102, KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K07246
N651.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N655.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N656.S	8	KO:K00156, KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N657.S	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N664	8	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778, KO:K10783
N667.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N675.S	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N693	8	KO:K00004, KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N698.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N712.S	8	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N714.S	8	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N734.S	8	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01734, KO:K03778, KO:K04020
N782.S	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N789.S	8	KO:K00156, KO:K00163, KO:K00627, KO:K00656, KO:K01067, KO:K01069, KO:K01442, KO:K01734
N792	8	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N794	8	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N817.S	8	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N844	8	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K04020
N851.S	8	KO:K00102, KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01734, KO:K04020
N876	8	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N1051	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01659, KO:K01734, KO:K03778
N402.S	7	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N403.S	7	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N414.S	7	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N416.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N433.S	7	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N448.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N451.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N460.S	7	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N462.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N466.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N470.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N479.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N480.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N484.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N511.S	7	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N518.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N520.S	7	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778

N524.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N542.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N580.S	7	KO:K00158, KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N587.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N611.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N612.S	7	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N625.S	7	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03366
N649.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N685.S	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N686.S	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N687	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N733.S	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N739.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N777.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N785	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N788	7	KO:K00116, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01734, KO:K10783
N793.S	7	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734
N795.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778, KO:K10783
N798.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N804	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N807	7	KO:K00043, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N815.S	7	KO:K00102, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N829	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N836.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N846	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N875	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N894	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N898.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N913.S	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01734, KO:K03417, KO:K07246
N935	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N936.S	7	KO:K00156, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N945.S	7	KO:K00102, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N961	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N972	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N988	7	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N995.S	7	KO:K00627, KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N1002.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N1019.S	6	KO:K00156, KO:K00656, KO:K01067, KO:K01069, KO:K01659, KO:K01734
N1023	6	KO:K00627, KO:K00656, KO:K01442, KO:K01734, KO:K03366, KO:K03778
N1070.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01069, KO:K01442, KO:K01734
N399.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N421.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N436.S	6	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N439.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N441.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N446.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N457.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N464.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778

N465.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N468.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N469.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N474.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N488.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N508.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N510.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N512.S	6	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N514.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N517.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N521.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N522.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03417, KO:K07246
N523.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N525.S	6	KO:K00043, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N528.S	6	KO:K00656, KO:K01069, KO:K01734, KO:K03417, KO:K03778, KO:K07246
N537.S	6	KO:K00102, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N539.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N546.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N548.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N572.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N577.S	6	KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N581.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N602.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N614.S	6	KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N617.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N621.S	6	KO:K00043, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N652.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N654.S	6	KO:K00116, KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N668.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N678.S	6	KO:K00156, KO:K00627, KO:K00656, KO:K01069, KO:K01659, KO:K01734
N681.S	6	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N710.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N741.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N769	6	KO:K00102, KO:K00656, KO:K01069, KO:K01659, KO:K01734, KO:K03778
N779	6	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N796.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N826.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N827.S	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N828	6	KO:K00158, KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734
N832.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N840.S	6	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03366
N843.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N845	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N849	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N858	6	KO:K00043, KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N878	6	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N928.S	6	KO:K00634, KO:K00929, KO:K01067, KO:K01442, KO:K01734, KO:K03778
N960.S	6	KO:K00634, KO:K00656, KO:K00929, KO:K01442, KO:K01734, KO:K03778

N1061	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N463.S	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N529.S	5	KO:K00656, KO:K01069, KO:K01659, KO:K01734, KO:K03778
N533.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N545	5	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N570.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N574.S	5	KO:K00627, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N578.S	5	KO:K00656, KO:K01069, KO:K01659, KO:K01734, KO:K03778
N579.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N585.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N589.S	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N609.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N610.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N616.S	5	KO:K00634, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N623.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01659, KO:K01734
N648.S	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N653.S	5	KO:K00043, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N665.S	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N672.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N689	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K10783
N730	5	KO:K00634, KO:K00656, KO:K01442, KO:K01734, KO:K03778
N737.S	5	KO:K00656, KO:K01069, KO:K01442, KO:K01734, KO:K03778
N797.S	5	KO:K00627, KO:K00656, KO:K01659, KO:K01734, KO:K03366
N800.S	5	KO:K00627, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N802	5	KO:K00043, KO:K00627, KO:K00656, KO:K01442, KO:K01734
N830.S	5	KO:K00627, KO:K00634, KO:K00656, KO:K01442, KO:K01734
N833.S	5	KO:K00102, KO:K00634, KO:K00656, KO:K01442, KO:K01734
N835.S	5	KO:K00634, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N841	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N842.S	5	KO:K00634, KO:K00929, KO:K01442, KO:K01734, KO:K03778
N852	5	KO:K00656, KO:K01442, KO:K01659, KO:K01734, KO:K03778
N859	5	KO:K00102, KO:K00656, KO:K01069, KO:K01734, KO:K03778
N860	5	KO:K00627, KO:K00656, KO:K01069, KO:K01734, KO:K03778
N880	5	KO:K00634, KO:K00656, KO:K01069, KO:K01442, KO:K01734
N885	5	KO:K00043, KO:K00656, KO:K01442, KO:K01659, KO:K01734
N925	5	KO:K00158, KO:K00627, KO:K00656, KO:K01442, KO:K01734
N983	5	KO:K00627, KO:K00656, KO:K01442, KO:K01734, KO:K03366
N1004.S	4	KO:K00656, KO:K01069, KO:K01734, KO:K03778
N1078.S	4	KO:K00158, KO:K00627, KO:K00656, KO:K01069
N1088	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N538.S	4	KO:K00656, KO:K01069, KO:K01734, KO:K03778
N604.S	4	KO:K00656, KO:K01659, KO:K01734, KO:K03778
N669.S	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N676.S	4	KO:K00656, KO:K01069, KO:K01734, KO:K03778
N690.S	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N692.S	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N695.S	4	KO:K00116, KO:K00656, KO:K01442, KO:K01734
N709	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734

N738.S	4	KO:K00656, KO:K01659, KO:K01734, KO:K03778
N775.S	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N776.S	4	KO:K00116, KO:K00656, KO:K01442, KO:K01734
N780.S	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N781	4	KO:K00656, KO:K01069, KO:K01442, KO:K01734
N808.S	4	KO:K00656, KO:K01069, KO:K01734, KO:K10783
N809.S	4	KO:K00656, KO:K01069, KO:K01659, KO:K01734
N811.S	4	KO:K00116, KO:K00656, KO:K01069, KO:K01734
N869.S	4	KO:K00627, KO:K00656, KO:K01442, KO:K01734
N871.S	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N881	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N907.S	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N927	4	KO:K00656, KO:K01067, KO:K01442, KO:K01734
N942	4	KO:K00102, KO:K00656, KO:K01442, KO:K01734
N947	4	KO:K00627, KO:K00656, KO:K01442, KO:K01734
N982	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N986.S	4	KO:K00656, KO:K01442, KO:K01734, KO:K03778
N987	4	KO:K00634, KO:K00656, KO:K01442, KO:K01734
N998.S	4	KO:K00656, KO:K01442, KO:K01659, KO:K01734
N1089	3	KO:K00656, KO:K01659, KO:K01734
N663.S	3	KO:K00656, KO:K01442, KO:K01734
N703.S	3	KO:K00656, KO:K01659, KO:K01734
N805.S	3	KO:K00656, KO:K01069, KO:K01442
N874.S	3	KO:K00656, KO:K01442, KO:K01734
N996	3	KO:K00656, KO:K01442, KO:K01734
N1092	2	KO:K01442, KO:K01734
N660.S	2	KO:K00656, KO:K01442

Table 22

Phylogenetic clades with alternative embodiments

Phylogenetic clade	ITEs in clade
clade_172	Bifidobacteriaceae genomosp. C1, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium infantis, Bifidobacterium kashiwanohense, Bifidobacterium longum, Bifidobacterium pseudocatenulatum, Bifidobacterium pseudolongum, Bifidobacterium scardovii, Bifidobacterium sp. HM2, Bifidobacterium sp. HMLN12, Bifidobacterium sp. M45, Bifidobacterium sp. MSX5B, Bifidobacterium sp. TM_7, Bifidobacterium thermophilum
clade_172i	Bifidobacteriaceae genomosp. C1, Bifidobacterium angulatum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium infantis, Bifidobacterium kashiwanohense, Bifidobacterium pseudocatenulatum, Bifidobacterium pseudolongum, Bifidobacterium scardovii, Bifidobacterium sp. HM2, Bifidobacterium sp. HMLN12, Bifidobacterium sp. M45, Bifidobacterium sp. MSX5B, Bifidobacterium sp. TM_7, Bifidobacterium thermophilum
clade_198	Lactobacillus casei, Lactobacillus paracasei, Lactobacillus zeae
clade_198i	Lactobacillus zeae

clade_260	Clostridium hylemonae, Clostridium scindens, Lachnospiraceae bacterium 5_1_57FAA
clade_260c	Clostridium hylemonae, Lachnospiraceae bacterium 5_1_57FAA
clade_260g	Clostridium hylemonae, Lachnospiraceae bacterium 5_1_57FAA
clade_260h	Clostridium hylemonae, Lachnospiraceae bacterium 5_1_57FAA
clade_262	Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, Ruminococcus torques
clade_262i	Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris
clade_309	Blautia coccoides, Blautia glucerasea, Blautia glucerasei, Blautia hansenii, Blautia luti, Blautia producta, Blautia schinkii, Blautia sp. M25, Blautia stercoris, Blautia wexlerae, Bryantella formatexigens, Clostridium coccoides, Eubacterium cellulosolvens, Lachnospiraceae bacterium 6_1_63FAA, Marvinbryantia formatexigens, Ruminococcus hansenii, Ruminococcus obeum, Ruminococcus sp. 5_1_39BFAA, Ruminococcus sp. K_1, Syntrophococcus sucromutans
clade_309c	Blautia coccoides, Blautia glucerasea, Blautia glucerasei, Blautia hansenii, Blautia luti, Blautia schinkii, Blautia sp. M25, Blautia stercoris, Blautia wexlerae, Bryantella formatexigens, Clostridium coccoides, Eubacterium cellulosolvens, Lachnospiraceae bacterium 6_1_63FAA, Marvinbryantia formatexigens, Ruminococcus hansenii, Ruminococcus obeum, Ruminococcus sp. 5_1_39BFAA, Ruminococcus sp. K_1, Syntrophococcus sucromutans
clade_309e	Blautia coccoides, Blautia glucerasea, Blautia glucerasei, Blautia hansenii, Blautia luti, Blautia schinkii, Blautia sp. M25, Blautia stercoris, Blautia wexlerae, Bryantella formatexigens, Clostridium coccoides, Eubacterium cellulosolvens, Lachnospiraceae bacterium 6_1_63FAA, Marvinbryantia formatexigens, Ruminococcus hansenii, Ruminococcus obeum, Ruminococcus sp. 5_1_39BFAA, Ruminococcus sp. K_1, Syntrophococcus sucromutans
clade_309g	Blautia coccoides, Blautia glucerasea, Blautia glucerasei, Blautia hansenii, Blautia luti, Blautia schinkii, Blautia sp. M25, Blautia stercoris, Blautia wexlerae, Bryantella formatexigens, Clostridium coccoides, Eubacterium cellulosolvens, Lachnospiraceae bacterium 6_1_63FAA, Marvinbryantia formatexigens, Ruminococcus hansenii, Ruminococcus obeum, Ruminococcus sp. 5_1_39BFAA, Ruminococcus sp. K_1, Syntrophococcus sucromutans
clade_309h	Blautia coccoides, Blautia glucerasea, Blautia glucerasei, Blautia hansenii, Blautia luti, Blautia schinkii, Blautia sp. M25, Blautia stercoris, Blautia wexlerae, Bryantella formatexigens, Clostridium coccoides, Eubacterium cellulosolvens, Lachnospiraceae bacterium 6_1_63FAA, Marvinbryantia formatexigens, Ruminococcus hansenii, Ruminococcus obeum, Ruminococcus sp. 5_1_39BFAA, Ruminococcus sp. K_1, Syntrophococcus sucromutans
clade_309i	Blautia coccoides, Blautia glucerasea, Blautia glucerasei, Blautia hansenii, Blautia luti, Blautia schinkii, Blautia sp. M25, Blautia stercoris, Blautia wexlerae, Bryantella formatexigens, Clostridium coccoides, Eubacterium cellulosolvens, Lachnospiraceae bacterium 6_1_63FAA, Marvinbryantia formatexigens, Ruminococcus hansenii, Ruminococcus sp. 5_1_39BFAA, Ruminococcus sp. K_1, Syntrophococcus sucromutans
clade_313	Lactobacillus antri, Lactobacillus coleohominis, Lactobacillus fermentum, Lactobacillus gastricus, Lactobacillus mucosae, Lactobacillus oris, Lactobacillus pontis, Lactobacillus reuteri, Lactobacillus sp. KLDS 1.0707, Lactobacillus sp. KLDS 1.0709, Lactobacillus sp. KLDS 1.0711, Lactobacillus sp. KLDS 1.0713, Lactobacillus sp. KLDS 1.0716, Lactobacillus sp. KLDS 1.0718, Lactobacillus sp. oral taxon 052, Lactobacillus vaginalis
clade_313f	Lactobacillus antri, Lactobacillus coleohominis, Lactobacillus fermentum, Lactobacillus gastricus, Lactobacillus mucosae, Lactobacillus oris, Lactobacillus pontis, Lactobacillus sp. KLDS 1.0707, Lactobacillus sp. KLDS 1.0709, Lactobacillus sp. KLDS 1.0711, Lactobacillus sp. KLDS 1.0713, Lactobacillus sp. KLDS 1.0716, Lactobacillus sp. KLDS 1.0718, Lactobacillus sp. oral taxon 052, Lactobacillus vaginalis
clade_325	Staphylococcus aureus, Staphylococcus auricularis, Staphylococcus capitis, Staphylococcus caprae, Staphylococcus carnosus, Staphylococcus cohnii, Staphylococcus condimenti, Staphylococcus epidermidis, Staphylococcus equorum, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus pasteuri, Staphylococcus pseudintermedius, Staphylococcus saccharolyticus, Staphylococcus saprophyticus, Staphylococcus sp. H292, Staphylococcus sp. H780, Staphylococcus sp. clone bottae7, Staphylococcus succinus, Staphylococcus warneri, Staphylococcus xylosus

clade_325f	Staphylococcus aureus, Staphylococcus auricularis, Staphylococcus capitis, Staphylococcus caprae, Staphylococcus carnosus, Staphylococcus cohnii, Staphylococcus condimenti, Staphylococcus epidermidis, Staphylococcus equorum, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus pseudintermedius, Staphylococcus saccharolyticus, Staphylococcus saprophyticus, Staphylococcus sp. H292, Staphylococcus sp. H780, Staphylococcus sp. clone bottae7, Staphylococcus succinus, Staphylococcus xylosus
clade_335	Bacteroides sp. 20_3, Bacteroides sp. 3_1_19, Bacteroides sp. 3_2_5, Parabacteroides distasonis, Parabacteroides goldsteinii, Parabacteroides gordonii, Parabacteroides sp. D13
clade_335i	Bacteroides sp. 20_3, Bacteroides sp. 3_1_19, Bacteroides sp. 3_2_5, Parabacteroides goldsteinii, Parabacteroides gordonii, Parabacteroides sp. D13
clade_351	Clostridium innocuum, Clostridium sp. HGF2
clade_351e	Clostridium sp. HGF2
clade_354	Clostridium bartlettii, Clostridium bifermentans, Clostridium ghonii, Clostridium glycolicum, Clostridium mayombei, Clostridium sordellii, Clostridium sp. MT4 E, Eubacterium tenue
clade_354e	Clostridium bartlettii, Clostridium ghonii, Clostridium glycolicum, Clostridium mayombei, Clostridium sordellii, Clostridium sp. MT4 E, Eubacterium tenue
clade_360	Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus, Ruminococcus sp. ID8
clade_360e	Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus
clade_360g	Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus
clade_360h	Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus
clade_360i	Dorea formicigenerans, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus, Ruminococcus sp. ID8
clade_378	Bacteroides barnesiae, Bacteroides coprocola, Bacteroides coprophilus, Bacteroides dorei, Bacteroides massiliensis, Bacteroides plebeius, Bacteroides sp. 3_1_33FAA, Bacteroides sp. 3_1_40A, Bacteroides sp. 4_3_47FAA, Bacteroides sp. 9_1_42FAA, Bacteroides sp. NB_8, Bacteroides vulgatus
clade_378e	Bacteroides barnesiae, Bacteroides coprocola, Bacteroides coprophilus, Bacteroides dorei, Bacteroides massiliensis, Bacteroides plebeius, Bacteroides sp. 3_1_33FAA, Bacteroides sp. 3_1_40A, Bacteroides sp. 4_3_47FAA, Bacteroides sp. 9_1_42FAA, Bacteroides sp. NB_8
clade_38	Bacteroides ovatus, Bacteroides sp. 1_1_30, Bacteroides sp. 2_1_22, Bacteroides sp. 2_2_4, Bacteroides sp. 3_1_23, Bacteroides sp. D1, Bacteroides sp. D2, Bacteroides sp. D22, Bacteroides xylanisolvens
clade_38e	Bacteroides sp. 1_1_30, Bacteroides sp. 2_1_22, Bacteroides sp. 2_2_4, Bacteroides sp. 3_1_23, Bacteroides sp. D1, Bacteroides sp. D2, Bacteroides sp. D22, Bacteroides xylanisolvens
clade_38i	Bacteroides sp. 1_1_30, Bacteroides sp. 2_1_22, Bacteroides sp. 2_2_4, Bacteroides sp. 3_1_23, Bacteroides sp. D1, Bacteroides sp. D2, Bacteroides sp. D22, Bacteroides xylanisolvens
clade_408	Anaerostipes caccae, Anaerostipes sp. 3_2_56FAA, Clostridiales bacterium 1_7_47FAA, Clostridiales sp. SM4_1, Clostridiales sp. SSC_2, Clostridium aerotolerans, Clostridium aldenense, Clostridium algidixylanolyticum, Clostridium amygdalinum, Clostridium asparagiforme, Clostridium bolteae, Clostridium celerecrescens, Clostridium citroniae, Clostridium clostridiiformes, Clostridium clostridioforme, Clostridium hathewayi, Clostridium indolis, Clostridium lavalense, Clostridium saccharolyticum, Clostridium sp. M62_1, Clostridium sp. SS2_1, Clostridium sphenoides, Clostridium symbiosum, Clostridium xylanolyticum, Eubacterium hadrum, Fusobacterium naviforme, Lachnospiraceae bacterium 3_1_57FAA, Lachnospiraceae bacterium 5_1_63FAA, Lachnospiraceae bacterium A4,

	Lachnospiraceae bacterium DJF VP30, Lachnospiraceae genomosp. C1, Moryella indoligenes
clade_408b	Anaerostipes caccae, Anaerostipes sp. 3_2_56FAA, Clostridiales bacterium 1_7_47FAA, Clostridiales sp. SM4_1, Clostridiales sp. SSC_2, Clostridium aerotolerans, Clostridium aldenense, Clostridium algidixylanolyticum, Clostridium amygdalinum, Clostridium asparagiforme, Clostridium bolteae, Clostridium celerecrescens, Clostridium citroniae, Clostridium clostridiiformes, Clostridium clostridioforme, Clostridium indolis, Clostridium lavalense, Clostridium saccharolyticum, Clostridium sp. M62_1, Clostridium sp. SS2_1, Clostridium sphenoides, Clostridium symbiosum, Clostridium xylanolyticum, Eubacterium hadrum, Fusobacterium naviforme, Lachnospiraceae bacterium 5_1_63FAA, Lachnospiraceae bacterium A4, Lachnospiraceae bacterium DJF VP30, Lachnospiraceae genomosp. C1, Moryella indoligenes
clade_408d	Anaerostipes caccae, Anaerostipes sp. 3_2_56FAA, Clostridiales sp. SM4_1, Clostridiales sp. SSC_2, Clostridium aerotolerans, Clostridium aldenense, Clostridium algidixylanolyticum, Clostridium amygdalinum, Clostridium celerecrescens, Clostridium citroniae, Clostridium clostridiiformes, Clostridium clostridioforme, Clostridium hathewayi, Clostridium lavalense, Clostridium saccharolyticum, Clostridium sp. M62_1, Clostridium sp. SS2_1, Clostridium sphenoides, Clostridium symbiosum, Clostridium xylanolyticum, Eubacterium hadrum, Fusobacterium naviforme, Lachnospiraceae bacterium 3_1_57FAA, Lachnospiraceae bacterium 5_1_63FAA, Lachnospiraceae bacterium A4, Lachnospiraceae bacterium DJF VP30, Lachnospiraceae genomosp. C1, Moryella indoligenes
clade_408f	Anaerostipes sp. 3_2_56FAA, Clostridiales bacterium 1_7_47FAA, Clostridiales sp. SM4_1, Clostridiales sp. SSC_2, Clostridium aerotolerans, Clostridium aldenense, Clostridium algidixylanolyticum, Clostridium amygdalinum, Clostridium asparagiforme, Clostridium bolteae, Clostridium celerecrescens, Clostridium citroniae, Clostridium clostridiiformes, Clostridium clostridioforme, Clostridium hathewayi, Clostridium lavalense, Clostridium saccharolyticum, Clostridium sp. M62_1, Clostridium sp. SS2_1, Clostridium sphenoides, Clostridium symbiosum, Clostridium xylanolyticum, Eubacterium hadrum, Fusobacterium naviforme, Lachnospiraceae bacterium 3_1_57FAA, Lachnospiraceae bacterium 5_1_63FAA, Lachnospiraceae bacterium A4, Lachnospiraceae bacterium DJF VP30, Lachnospiraceae genomosp. C1, Moryella indoligenes
clade_408g	Anaerostipes caccae, Anaerostipes sp. 3_2_56FAA, Clostridiales sp. SM4_1, Clostridiales sp. SSC_2, Clostridium aerotolerans, Clostridium aldenense, Clostridium algidixylanolyticum, Clostridium amygdalinum, Clostridium celerecrescens, Clostridium citroniae, Clostridium clostridiiformes, Clostridium clostridioforme, Clostridium lavalense, Clostridium saccharolyticum, Clostridium sp. M62_1, Clostridium sp. SS2_1, Clostridium sphenoides, Clostridium symbiosum, Clostridium xylanolyticum, Eubacterium hadrum, Fusobacterium naviforme, Lachnospiraceae bacterium 5_1_63FAA, Lachnospiraceae bacterium A4, Lachnospiraceae bacterium DJF VP30, Lachnospiraceae genomosp. C1, Moryella indoligenes
clade_408h	Anaerostipes caccae, Anaerostipes sp. 3_2_56FAA, Clostridiales sp. SM4_1, Clostridiales sp. SSC_2, Clostridium aerotolerans, Clostridium aldenense, Clostridium algidixylanolyticum, Clostridium amygdalinum, Clostridium celerecrescens, Clostridium citroniae, Clostridium clostridiiformes, Clostridium clostridioforme, Clostridium lavalense, Clostridium saccharolyticum, Clostridium sp. M62_1, Clostridium sp. SS2_1, Clostridium sphenoides, Clostridium symbiosum, Clostridium xylanolyticum, Eubacterium hadrum, Fusobacterium naviforme, Lachnospiraceae bacterium 5_1_63FAA, Lachnospiraceae bacterium A4, Lachnospiraceae bacterium DJF VP30, Lachnospiraceae genomosp. C1, Moryella indoligenes
clade_420	Barnesiella intestinihominis, Barnesiella viscericola, Parabacteroides sp. NS31_3, Porphyromonadaceae bacterium NML 060648, Tannerella forsythia, Tannerella sp. 6_1_58FAA_CT1
clade_420f	Barnesiella viscericola, Parabacteroides sp. NS31_3, Porphyromonadaceae bacterium NML 060648, Tannerella forsythia, Tannerella sp. 6_1_58FAA_CT1
clade_444	Butyrivibrio fibrisolvens, Eubacterium rectale, Eubacterium sp. oral clone GI038, Lachnobacterium bovis, Roseburia cecicola, Roseburia faecalis, Roseburia faecis, Roseburia hominis, Roseburia intestinalis, Roseburia inulinivorans, Roseburia sp. 11SE37, Roseburia sp. 11SE38, Shuttleworthia satelles, Shuttleworthia sp. MSX8B, Shuttleworthia sp.

	oral taxon G69
clade_444i	Butyrivibrio fibrisolvens, Eubacterium sp. oral clone GI038, Lachnobacterium bovis, Roseburia cecicola, Roseburia faecis, Roseburia hominis, Roseburia inulinivorans, Roseburia sp. 11SE37, Roseburia sp. 11SE38, Shuttleworthia satelles, Shuttleworthia sp. MSX8B, Shuttleworthia sp. oral taxon G69
clade_478	Faecalibacterium prausnitzii, Gemmiger formicilis, Subdoligranulum variabile
clade_478i	Gemmiger formicilis, Subdoligranulum variabile
clade_479	Clostridiaceae bacterium JC13, Clostridium sp. MLG055, Erysipelotrichaceae bacterium 3_1_53
clade_479c	Clostridium sp. MLG055, Erysipelotrichaceae bacterium 3_1_53
clade_479g	Clostridium sp. MLG055, Erysipelotrichaceae bacterium 3_1_53
clade_479h	Clostridium sp. MLG055, Erysipelotrichaceae bacterium 3_1_53
clade_481	Clostridium cocleatum, Clostridium ramosum, Clostridium saccharogumia, Clostridium spiroforme, Coprobacillus sp. D7
clade_481a	Clostridium cocleatum, Clostridium spiroforme, Coprobacillus sp. D7
clade_481b	Clostridium cocleatum, Clostridium ramosum, Clostridium spiroforme, Coprobacillus sp. D7
clade_481c	Clostridium cocleatum, Clostridium saccharogumia, Clostridium spiroforme, Coprobacillus sp. D7
clade_481g	Clostridium cocleatum, Clostridium spiroforme, Coprobacillus sp. D7
clade_481h	Clostridium cocleatum, Clostridium spiroforme, Coprobacillus sp. D7
clade_481i	Clostridium ramosum, Clostridium saccharogumia, Clostridium spiroforme, Coprobacillus sp. D7
clade_497	Abiotrophia para_adiacens, Carnobacterium divergens, Carnobacterium maltaromaticum, Enterococcus avium, Enterococcus caccae, Enterococcus casseliflavus, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus gallinarum, Enterococcus gilvus, Enterococcus hawaiiensis, Enterococcus hirae, Enterococcus italicus, Enterococcus mundtii, Enterococcus raffinosus, Enterococcus sp. BV2CASA2, Enterococcus sp. CCRI 16620, Enterococcus sp. F95, Enterococcus sp. RfL6, Enterococcus thailandicus, Fusobacterium canifelinum, Fusobacterium genomosp. C1, Fusobacterium genomosp. C2, Fusobacterium nucleatum, Fusobacterium periodonticum, Fusobacterium sp. 11_3_2, Fusobacterium sp. 1_1_41FAA, Fusobacterium sp. 2_1_31, Fusobacterium sp. 3_1_27, Fusobacterium sp. 3_1_33, Fusobacterium sp. 3_1_36A2, Fusobacterium sp. AC18, Fusobacterium sp. ACB2, Fusobacterium sp. AS2, Fusobacterium sp. CM1, Fusobacterium sp. CM21, Fusobacterium sp. CM22, Fusobacterium sp. oral clone ASCF06, Fusobacterium sp. oral clone ASCF11, Granulicatella adiacens, Granulicatella elegans, Granulicatella paradiacens, Granulicatella sp. oral clone ASC02, Granulicatella sp. oral clone ASCA05, Granulicatella sp. oral clone ASCB09, Granulicatella sp. oral clone ASCG05, Tetragenococcus halophilus, Tetragenococcus koreensis, Vagococcus fluvialis
clade_497e	Abiotrophia para_adiacens, Carnobacterium divergens, Carnobacterium maltaromaticum, Enterococcus avium, Enterococcus caccae, Enterococcus casseliflavus, Enterococcus durans, Enterococcus faecium, Enterococcus gallinarum, Enterococcus gilvus, Enterococcus hawaiiensis, Enterococcus hirae, Enterococcus italicus, Enterococcus mundtii, Enterococcus raffinosus, Enterococcus sp. BV2CASA2, Enterococcus sp. CCRI 16620, Enterococcus sp. F95, Enterococcus sp. RfL6, Enterococcus thailandicus, Fusobacterium canifelinum, Fusobacterium genomosp. C1, Fusobacterium genomosp. C2, Fusobacterium nucleatum, Fusobacterium periodonticum, Fusobacterium sp. 11_3_2, Fusobacterium sp. 1_1_41FAA, Fusobacterium sp. 2_1_31, Fusobacterium sp. 3_1_27, Fusobacterium sp. 3_1_33, Fusobacterium sp. 3_1_36A2, Fusobacterium sp. AC18, Fusobacterium sp. ACB2, Fusobacterium sp. AS2, Fusobacterium sp. CM1, Fusobacterium sp. CM21, Fusobacterium sp. CM22, Fusobacterium sp. oral clone ASCF06, Fusobacterium sp. oral clone ASCF11, Granulicatella adiacens, Granulicatella elegans, Granulicatella paradiacens, Granulicatella sp. oral clone ASC02, Granulicatella sp. oral clone ASCA05, Granulicatella sp. oral clone ASCB09, Granulicatella sp. oral clone ASCG05, Tetragenococcus halophilus, Tetragenococcus koreensis, Vagococcus fluvialis

clade_497f	Abiotrophia para_adiacens, Carnobacterium divergens, Carnobacterium maltaromaticum, Enterococcus avium, Enterococcus caccae, Enterococcus casseliflavus, Enterococcus faecalis, Enterococcus gallinarum, Enterococcus gilvus, Enterococcus hawaiiensis, Enterococcus italicus, Enterococcus mundtii, Enterococcus raffinosus, Enterococcus sp. BV2CASA2, Enterococcus sp. CCRI 16620, Enterococcus sp. F95, Enterococcus sp. RfL6, Enterococcus thailandicus, Fusobacterium canifelinum, Fusobacterium genomosp. C1, Fusobacterium genomosp. C2, Fusobacterium nucleatum, Fusobacterium periodonticum, Fusobacterium sp. 11_3_2, Fusobacterium sp. 1_1_41FAA, Fusobacterium sp. 2_1_31, Fusobacterium sp. 3_1_27, Fusobacterium sp. 3_1_33, Fusobacterium sp. 3_1_36A2, Fusobacterium sp. AC18, Fusobacterium sp. ACB2, Fusobacterium sp. AS2, Fusobacterium sp. CM1, Fusobacterium sp. CM21, Fusobacterium sp. CM22, Fusobacterium sp. oral clone ASCF06, Fusobacterium sp. oral clone ASCF11, Granulicatella adiacens, Granulicatella elegans, Granulicatella paradiacens, Granulicatella sp. oral clone ASC02, Granulicatella sp. oral clone ASCA05, Granulicatella sp. oral clone ASCB09, Granulicatella sp. oral clone ASCG05, Tetragenococcus halophilus, Tetragenococcus koreensis, Vagococcus fluvialis
clade_512	Eubacterium barkeri, Eubacterium callanderi, Eubacterium limosum, Pseudoramibacter alactolyticus
clade_512i	Eubacterium barkeri, Eubacterium callanderi, Pseudoramibacter alactolyticus
clade_516	Anaerotruncus colihominis, Clostridium methylpentosum, Clostridium sp. YIT 12070, Hydrogenoanaerobacterium saccharovorans, Ruminococcus albus, Ruminococcus flavefaciens
clade_516c	Clostridium methylpentosum, Clostridium sp. YIT 12070, Hydrogenoanaerobacterium saccharovorans, Ruminococcus albus, Ruminococcus flavefaciens
clade_516g	Clostridium methylpentosum, Clostridium sp. YIT 12070, Hydrogenoanaerobacterium saccharovorans, Ruminococcus albus, Ruminococcus flavefaciens
clade_516h	Clostridium methylpentosum, Clostridium sp. YIT 12070, Hydrogenoanaerobacterium saccharovorans, Ruminococcus albus, Ruminococcus flavefaciens
clade_519	Eubacterium ventriosum
clade_522	Bacteroides galacturonicus, Eubacterium eligens, Lachnospira multipara, Lachnospira pectinoschiza, Lactobacillus rogosae
clade_522i	Bacteroides galacturonicus, Lachnospira multipara, Lachnospira pectinoschiza, Lactobacillus rogosae
clade_553	Collinsella aerofaciens, Collinsella intestinalis, Collinsella stercoris, Collinsella tanakaei
clade_553i	Collinsella intestinalis, Collinsella stercoris, Collinsella tanakaei
clade_566	Adlercreutzia equolifaciens, Coriobacteriaceae bacterium JC110, Coriobacteriaceae bacterium phI, Cryptobacterium curtum, Eggerthella lenta, Eggerthella sinensis, Eggerthella sp. 1_3_56FAA, Eggerthella sp. HGA1, Eggerthella sp. YY7918, Gordonibacter pamelaeae, Slackia equolifaciens, Slackia exigua, Slackia faecicanis, Slackia heliotrinireducens, Slackia isoflavoniconvertens, Slackia piriformis, Slackia sp. NATTS, Streptomyces albus
clade_566f	Coriobacteriaceae bacterium JC110, Coriobacteriaceae bacterium phI, Cryptobacterium curtum, Eggerthella lenta, Eggerthella sinensis, Eggerthella sp. 1_3_56FAA, Eggerthella sp. HGA1, Eggerthella sp. YY7918, Gordonibacter pamelaeae, Slackia equolifaciens, Slackia exigua, Slackia faecicanis, Slackia heliotrinireducens, Slackia isoflavoniconvertens, Slackia piriformis, Slackia sp. NATTS, Streptomyces albus
clade_572	Butyricicoccus pullicaecorum, Eubacterium desmolans, Papillibacter cinnamivorans, Sporobacter termitidis
clade_572i	Butyricicoccus pullicaecorum, Papillibacter cinnamivorans, Sporobacter termitidis
clade_65	Bacteroides faecis, Bacteroides fragilis, Bacteroides nordii, Bacteroides salyersiae, Bacteroides sp. 1_1_14, Bacteroides sp. 1_1_6, Bacteroides sp. 2_1_56FAA, Bacteroides sp. AR29, Bacteroides sp. B2, Bacteroides thetaiotaomicron
clade_65e	Bacteroides faecis, Bacteroides fragilis, Bacteroides nordii, Bacteroides salyersiae, Bacteroides sp. 1_1_14, Bacteroides sp. 1_1_6, Bacteroides sp. 2_1_56FAA, Bacteroides sp. AR29, Bacteroides sp. B2

clade_92	Actinobacillus actinomycetemcomitans, Actinobacillus succinogenes, Aggregatibacter actinomycetemcomitans, Aggregatibacter aphrophilus, Aggregatibacter segnis, Averyella dalhousiensis, Bisgaard Taxon, Buchnera aphidicola, Cedecea davisae, Citrobacter amalonaticus, Citrobacter braakii, Citrobacter farmeri, Citrobacter freundii, Citrobacter gillenii, Citrobacter koseri, Citrobacter murliniae, Citrobacter rodentium, Citrobacter sedlakii, Citrobacter sp. 30_2, Citrobacter sp. KMSI_3, Citrobacter werkmanii, Citrobacter youngae, Cronobacter malonaticus, Cronobacter sakazakii, Cronobacter turicensis, Enterobacter aerogenes, Enterobacter asburiae, Enterobacter cancerogenus, Enterobacter cloacae, Enterobacter cowanii, Enterobacter hormaechei, Enterobacter sp. 247BMC, Enterobacter sp. 638, Enterobacter sp. JC163, Enterobacter sp. SCSS, Enterobacter sp. TSE38, Enterobacteriaceae bacterium 9_2_54FAA, Enterobacteriaceae bacterium CF01Ent_1, Enterobacteriaceae bacterium Smarlab 3302238, Escherichia albertii, Escherichia coli, Escherichia fergusonii, Escherichia hermannii, Escherichia sp. 1_1_43, Escherichia sp. 4_1_40B, Escherichia sp. B4, Escherichia vulneris, Ewingella americana, Haemophilus genomosp. P2 oral clone MB3_C24, Haemophilus genomosp. P3 oral clone MB3_C38, Haemophilus sp. oral clone JM053, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella sp. AS10, Klebsiella sp. Co9935, Klebsiella sp. OBRC7, Klebsiella sp. SP_BA, Klebsiella sp. SRC_DSD1, Klebsiella sp. SRC_DSD11, Klebsiella sp. SRC_DSD12, Klebsiella sp. SRC_DSD15, Klebsiella sp. SRC_DSD2, Klebsiella sp. SRC_DSD6, Klebsiella sp. enrichment culture clone SRC_DSD25, Klebsiella variicola, Kluyvera ascorbata, Kluyvera cryocrescens, Leminorella grimontii, Leminorella richardii, Pantoea agglomerans, Pantoea ananatis, Pantoea brenneri, Pantoea citrea, Pantoea conspicua, Pantoea septica, Pasteurella dagmatis, Pasteurella multocida, Plesiomonas shigelloides, Raoultella ornithinolytica, Raoultella planticola, Raoultella terrigena, Salmonella bongori, Salmonella enterica, Salmonella typhimurium, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Serratia odorifera, Serratia proteamaculans, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Tatumella ptyseos, Trabulsiella guamensis, Yersinia aldovae, Yersinia aleksiciae, Yersinia bercovieri, Yersinia enterocolitica, Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia mollaretii, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia rohdei, Yokenella regensburgei
clade_92e	Actinobacillus actinomycetemcomitans, Actinobacillus succinogenes, Aggregatibacter actinomycetemcomitans, Aggregatibacter aphrophilus, Aggregatibacter segnis, Averyella dalhousiensis, Bisgaard Taxon, Buchnera aphidicola, Cedecea davisae, Citrobacter amalonaticus, Citrobacter braakii, Citrobacter farmeri, Citrobacter freundii, Citrobacter gillenii, Citrobacter koseri, Citrobacter murliniae, Citrobacter rodentium, Citrobacter sedlakii, Citrobacter sp. 30_2, Citrobacter sp. KMSI_3, Citrobacter werkmanii, Citrobacter youngae, Cronobacter malonaticus, Cronobacter sakazakii, Cronobacter turicensis, Enterobacter aerogenes, Enterobacter asburiae, Enterobacter cancerogenus, Enterobacter cloacae, Enterobacter cowanii, Enterobacter hormaechei, Enterobacter sp. 247BMC, Enterobacter sp. 638, Enterobacter sp. JC163, Enterobacter sp. SCSS, Enterobacter sp. TSE38, Enterobacteriaceae bacterium 9_2_54FAA, Enterobacteriaceae bacterium CF01Ent_1, Enterobacteriaceae bacterium Smarlab 3302238, Escherichia albertii, Escherichia fergusonii, Escherichia hermannii, Escherichia sp. 1_1_43, Escherichia sp. 4_1_40B, Escherichia sp. B4, Escherichia vulneris, Ewingella americana, Haemophilus genomosp. P2 oral clone MB3_C24, Haemophilus genomosp. P3 oral clone MB3_C38, Haemophilus sp. oral clone JM053, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella sp. AS10, Klebsiella sp. Co9935, Klebsiella sp. OBRC7, Klebsiella sp. SP_BA, Klebsiella sp. SRC_DSD1, Klebsiella sp. SRC_DSD11, Klebsiella sp. SRC_DSD12, Klebsiella sp. SRC_DSD15, Klebsiella sp. SRC_DSD2, Klebsiella sp. SRC_DSD6, Klebsiella sp. enrichment culture clone SRC_DSD25, Klebsiella variicola, Kluyvera ascorbata, Kluyvera cryocrescens, Leminorella grimontii, Leminorella richardii, Pantoea agglomerans, Pantoea ananatis, Pantoea brenneri, Pantoea citrea, Pantoea conspicua, Pantoea septica, Pasteurella dagmatis, Pasteurella multocida, Plesiomonas shigelloides, Raoultella ornithinolytica, Raoultella planticola, Raoultella terrigena, Salmonella bongori, Salmonella enterica, Salmonella typhimurium, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Serratia odorifera, Serratia proteamaculans, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Tatumella ptyseos, Trabulsiella guamensis, Yersinia aldovae, Yersinia aleksiciae, Yersinia bercovieri, Yersinia enterocolitica, Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia mollaretii, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia rohdei, Yokenella regensburgei

clade_92i	Actinobacillus actinomycetemcomitans, Actinobacillus succinogenes, Aggregatibacter actinomycetemcomitans, Aggregatibacter aphrophilus, Aggregatibacter segnis, Averyella dalhousiensis, Bisgaard Taxon, Buchnera aphidicola, Cedecea davisae, Citrobacter amalonaticus, Citrobacter braakii, Citrobacter farmeri, Citrobacter freundii, Citrobacter gillenii, Citrobacter koseri, Citrobacter murliniae, Citrobacter rodentium, Citrobacter sedlakii, Citrobacter sp. 30_2, Citrobacter sp. KMSI_3, Citrobacter werkmanii, Citrobacter youngae, Cronobacter malonaticus, Cronobacter sakazakii, Cronobacter turicensis, Enterobacter aerogenes, Enterobacter asburiae, Enterobacter cancerogenus, Enterobacter cloacae, Enterobacter cowanii, Enterobacter hormaechei, Enterobacter sp. 247BMC, Enterobacter sp. 638, Enterobacter sp. JC163, Enterobacter sp. SCSS, Enterobacter sp. TSE38, Enterobacteriaceae bacterium 9_2_54FAA, Enterobacteriaceae bacterium CF01Ent_1, Enterobacteriaceae bacterium Smarlab 3302238, Escherichia albertii, Escherichia fergusonii, Escherichia hermannii, Escherichia sp. 1_1_43, Escherichia sp. 4_1_40B, Escherichia sp. B4, Escherichia vulneris, Ewingella americana, Haemophilus genomosp. P2 oral clone MB3_C24, Haemophilus genomosp. P3 oral clone MB3_C38, Haemophilus sp. oral clone JM053, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella sp. AS10, Klebsiella sp. Co9935, Klebsiella sp. OBRC7, Klebsiella sp. SP_BA, Klebsiella sp. SRC_DSD1, Klebsiella sp. SRC_DSD11, Klebsiella sp. SRC_DSD12, Klebsiella sp. SRC_DSD15, Klebsiella sp. SRC_DSD2, Klebsiella sp. SRC_DSD6, Klebsiella sp. enrichment culture clone SRC_DSD25, Klebsiella variicola, Kluyvera ascorbata, Kluyvera cryocrescens, Leminorella grimontii, Leminorella richardii, Pantoea agglomerans, Pantoea ananatis, Pantoea brenneri, Pantoea citrea, Pantoea conspicua, Pantoea septica, Pasteurella dagmatis, Pasteurella multocida, Plesiomonas shigelloides, Raoultella ornithinolytica, Raoultella planticola, Raoultella terrigena, Salmonella bongori, Salmonella enterica, Salmonella typhimurium, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Serratia odorifera, Serratia proteamaculans, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Tatumella ptyseos, Trabulsiella guamensis, Yersinia aldovae, Yersinia aleksiciae, Yersinia bercovieri, Yersinia enterocolitica, Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia mollaretii, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia rohdei, Yokenella regensburgei
clade_96	Clostridium oroticum, Clostridium sp. D5, Eubacterium contortum, Eubacterium fissicatena
clade_96g	Clostridium oroticum, Clostridium sp. D5, Eubacterium fissicatena
clade_96h	Clostridium oroticum, Clostridium sp. D5, Eubacterium fissicatena
clade_98	Okadaella gastrococcus, Streptococcus agalactiae, Streptococcus alactolyticus, Streptococcus australis, Streptococcus bovis, Streptococcus canis, Streptococcus constellatus, Streptococcus cristatus, Streptococcus dysgalactiae, Streptococcus equi, Streptococcus equinus, Streptococcus gallolyticus, Streptococcus genomosp. C1, Streptococcus genomosp. C2, Streptococcus genomosp. C3, Streptococcus genomosp. C4, Streptococcus genomosp. C5, Streptococcus genomosp. C6, Streptococcus genomosp. C7, Streptococcus genomosp. C8, Streptococcus gordonii, Streptococcus infantarius, Streptococcus infantis, Streptococcus intermedius, Streptococcus lutetiensis, Streptococcus massiliensis, Streptococcus mitis, Streptococcus oligofermentans, Streptococcus oralis, Streptococcus parasanguinis, Streptococcus pasteurianus, Streptococcus peroris, Streptococcus pneumoniae, Streptococcus porcinus, Streptococcus pseudopneumoniae, Streptococcus pseudoporcinus, Streptococcus pyogenes, Streptococcus ratti, Streptococcus salivarius, Streptococcus sanguinis, Streptococcus sinensis, Streptococcus sp. 2285_97, Streptococcus sp. 2_1_36FAA, Streptococcus sp. ACS2, Streptococcus sp. AS20, Streptococcus sp. BS35a, Streptococcus sp. C150, Streptococcus sp. CM6, Streptococcus sp. ICM10, Streptococcus sp. ICM12, Streptococcus sp. ICM2, Streptococcus sp. ICM4, Streptococcus sp. ICM45, Streptococcus sp. M143, Streptococcus sp. M334, Streptococcus sp. oral clone ASB02, Streptococcus sp. oral clone ASCA03, Streptococcus sp. oral clone ASCA04, Streptococcus sp. oral clone ASCA09, Streptococcus sp. oral clone ASCB04, Streptococcus sp. oral clone ASCB06, Streptococcus sp. oral clone ASCC04, Streptococcus sp. oral clone ASCC05, Streptococcus sp. oral clone ASCC12, Streptococcus sp. oral clone ASCD01, Streptococcus sp. oral clone ASCD09, Streptococcus sp. oral clone ASCD10, Streptococcus sp. oral clone ASCE03, Streptococcus sp. oral clone ASCE04, Streptococcus sp. oral clone ASCE05, Streptococcus sp. oral clone ASCE06, Streptococcus sp. oral clone ASCE09, Streptococcus sp. oral clone ASCE10, Streptococcus sp. oral clone ASCE12, Streptococcus sp. oral clone ASCF05, Streptococcus sp. oral clone ASCF07, Streptococcus sp. oral clone ASCF09, Streptococcus sp. oral clone ASCG04, Streptococcus sp. oral clone BW009, Streptococcus sp. oral clone CH016, Streptococcus sp. oral clone GK051, Streptococcus sp. oral clone GM006, Streptococcus sp. oral clone P2PA_41 P2, Streptococcus sp. oral clone P4PA_30 P4, Streptococcus sp. oral taxon 071, Streptococcus sp. oral taxon G59, Streptococcus sp. oral taxon G62, Streptococcus sp. oral taxon G63, Streptococcus suis, Streptococcus thermophilus,

	Streptococcus uberis, Streptococcus urinalis, Streptococcus vestibularis, Streptococcus viridans, Synergistetes bacterium oral clone 03 5 D05
clade_98i	Okadaella gastrococcus, Streptococcus agalactiae, Streptococcus alactolyticus, Streptococcus australis, Streptococcus bovis, Streptococcus canis, Streptococcus constellatus, Streptococcus cristatus, Streptococcus dysgalactiae, Streptococcus equi, Streptococcus equinus, Streptococcus gallolyticus, Streptococcus genomosp. C1, Streptococcus genomosp. C2, Streptococcus genomosp. C3, Streptococcus genomosp. C4, Streptococcus genomosp. C5, Streptococcus genomosp. C6, Streptococcus genomosp. C7, Streptococcus genomosp. C8, Streptococcus gordonii, Streptococcus infantarius, Streptococcus infantis, Streptococcus intermedius, Streptococcus lutetiensis, Streptococcus massiliensis, Streptococcus oligofermentans, Streptococcus oralis, Streptococcus parasanguinis, Streptococcus pasteurianus, Streptococcus peroris, Streptococcus pneumoniae, Streptococcus porcinus, Streptococcus pseudopneumoniae, Streptococcus pseudoporcinus, Streptococcus pyogenes, Streptococcus ratti, Streptococcus salivarius, Streptococcus sanguinis, Streptococcus sinensis, Streptococcus sp. 2285_97, Streptococcus sp. 2_1_36FAA, Streptococcus sp. ACS2, Streptococcus sp. AS20, Streptococcus sp. BS35a, Streptococcus sp. C150, Streptococcus sp. CM6, Streptococcus sp. ICM10, Streptococcus sp. ICM12, Streptococcus sp. ICM2, Streptococcus sp. ICM4, Streptococcus sp. ICM45, Streptococcus sp. M143, Streptococcus sp. M334, Streptococcus sp. oral clone ASB02, Streptococcus sp. oral clone ASCA03, Streptococcus sp. oral clone ASCA04, Streptococcus sp. oral clone ASCA09, Streptococcus sp. oral clone ASCB04, Streptococcus sp. oral clone ASCB06, Streptococcus sp. oral clone ASCC04, Streptococcus sp. oral clone ASCC05, Streptococcus sp. oral clone ASCC12, Streptococcus sp. oral clone ASCD01, Streptococcus sp. oral clone ASCD09, Streptococcus sp. oral clone ASCD10, Streptococcus sp. oral clone ASCE03, Streptococcus sp. oral clone ASCE04, Streptococcus sp. oral clone ASCE05, Streptococcus sp. oral clone ASCE06, Streptococcus sp. oral clone ASCE09, Streptococcus sp. oral clone ASCE10, Streptococcus sp. oral clone ASCE12, Streptococcus sp. oral clone ASCF05, Streptococcus sp. oral clone ASCF07, Streptococcus sp. oral clone ASCF09, Streptococcus sp. oral clone ASCG04, Streptococcus sp. oral clone BW009, Streptococcus sp. oral clone CH016, Streptococcus sp. oral clone GK051, Streptococcus sp. oral clone GM006, Streptococcus sp. oral clone P2PA_41 P2, Streptococcus sp. oral clone P4PA_30 P4, Streptococcus sp. oral taxon 071, Streptococcus sp. oral taxon G59, Streptococcus sp. oral taxon G62, Streptococcus sp. oral taxon G63, Streptococcus suis, Streptococcus thermophilus, Streptococcus uberis, Streptococcus urinalis, Streptococcus vestibularis, Streptococcus viridans, Synergistetes bacterium oral clone 03 5 D05

CLAIMS

1. A method for treating, preventing, or reducing the severity of a disorder selected from the group consisting of Clostridium difficile Associated Diarrhea (CDAD), Type 2 Diabetes, Obesity, Irritable Bowel Disease (IBD), colonization with a pathogen or pathobiont, and infection with a drug-resistant pathogen or pathobiont, comprising: administering to a mammalian subject in need thereof an effective amount of a therapeutic bacterial composition, said therapeutic bacterial composition comprising a plurality of isolated bacteria or a purified bacterial preparation, the plurality of isolated bacteria or the purified bacterial preparation capable of forming a network ecology selected from the group consisting of N262.S, N290.S, N284.S, N271.S, N282.S, N288.S, N302.S, N279.S, N310.S, N323.S, N331.S, N332.S, N301.S, N312.S, N339.S, N325.S, N340.S, N341.S, N346.S, N338.S, N336.S, N345.S, N355.S, N356.S, N343.S, N329.S, N361.S, N353.S, N381.S, N344.S, N352.S, N357.S, N358.S, N369.S, N372.S, N375.S, N380.S, N374.S, N377.S, N368.S, N370.S, N373.S, N376.S, N389.S, N394.S, N431.S, N434.S, N390.S, N397.S, N387.S, N440.S, N396.S, N399.S, N403.S, N414.S, N430.S, N432.S, N436.S, N437.S, N457.S, N545, N386.S, N402.S, N405.S, N415.S, N421.S, N422.S, N423.S, N458.S, N459.S, N493.S, N416.S, N439.S, N447.S, N490.S, N526, N429.S, N433.S, N448.S, N488.S, N508.S, N509.S, N510.S, N511.S, N408.S, N446.S, N451.S, N474.S, N520.S, N521.S, N535.S, N516.S, N463.S, N518.S, N586, N450.S, N465.S, N519.S, N537.S, N419.S, N468.S, N477.S, N514.S, N382.S, N460.S, N462.S, N512.S, N517.S, N523.S, N547.S, N548.S, N577.S, N581.S, N585.S, N616.S, N466.S, N469.S, N480.S, N482.S, N484.S, N515.S, N533.S, N709, N730, N478.S, N572.S, N400.S, N543.S, N582.S, N621.S, N689, N769, N481.S, N525.S, N528.S, N534.S, N574.S, N580.S, N590.S, N591.S, N597.S, N664, N693, N530.S, N687, N470.S, N529.S, N539.S, N546.S, N570.S, N579.S, N602.S, N614.S, N648.S, N652.S, N655.S, N672.S, N681.S, N690.S, N692.S, N698.S, N737.S, N738.S, N785, N841, N878, N880, N881, N987, N988, N996, N1061, N479.S, N538.S, N542.S, N578.S, N609.S, N611.S, N617.S, N666.S, N675.S, N682.S, N844, N845, N846, N852, N876, N982, N1008, N649.S, N657.S, N678.S, N686.S, N710.S, N522.S, N651.S, N653.S, N654.S, N680.S, N712.S, N792, N802, N804, N807, N849, N858, N859, N875, N885, N942, N961, N972, N1051, N587.S, N589.S, N612.S, N625.S, N656.S, N714.S, N779, N781, N828, N829, N860, N894, N925, N927, N935, N947, N983, N1023, N441.S, N584.S, N794, N788, N524.S, N604.S, N610.S, N623.S, N663.S, N669.S, N676.S, N703.S, N775.S, N777.S, N780.S, N817.S, N827.S, N836.S, N871.S, N874.S, N898.S, N907.S, N998.S, N1088, N1089, N660.S, N665.S, N667.S, N733.S, N734.S, N739.S, N741.S, N782.S,

N789.S, N796.S, N798.S, N800.S, N809.S, N816.S, N842.S, N843.S, N869.S, N986.S, N995.S, N1002.S, N1004.S, N1019.S, N1093, N668.S, N685.S, N835.S, N851.S, N464.S, N695.S, N776.S, N793.S, N815.S, N833.S, N891.S, N1070.S, N1092, N795.S, N797.S, N808.S, N811.S, N826.S, N830.S, N832.S, N840.S, N945.S, N960.S, N968.S, N1091, N805.S, N822.S, N928.S, N936.S, N1078.S, and N913.S.

2. The method of claim 1, wherein said therapeutic bacterial composition comprises at least one bacterial entity, wherein said bacterial entity is capable of forming the network ecology in combination with one more bacterial entities present in the gastrointestinal tract of the mammalian subject at the time of the administering or thereafter.

3. The method of claim 1, wherein the network ecology is selected from the group consisting of N1008, N1023, N1051, N1061, N1070.S, N1088, N1089, N1092, N381.S, N382.S, N387.S, N399.S, N400.S, N402.S, N403.S, N414.S, N429.S, N430.S, N432.S, N433.S, N436.S, N437.S, N439.S, N441.S, N447.S, N448.S, N457.S, N460.S, N462.S, N463.S, N464.S, N470.S, N474.S, N488.S, N490.S, N493.S, N508.S, N509.S, N510.S, N511.S, N512.S, N514.S, N515.S, N517.S, N518.S, N519.S, N520.S, N523.S, N524.S, N529.S, N539.S, N543.S, N546.S, N547.S, N548.S, N570.S, N574.S, N577.S, N579.S, N580.S, N582.S, N584.S, N585.S, N589.S, N591.S, N597.S, N602.S, N604.S, N609.S, N610.S, N611.S, N612.S, N614.S, N616.S, N621.S, N623.S, N625.S, N648.S, N651.S, N652.S, N653.S, N654.S, N655.S, N660.S, N663.S, N664, N665.S, N666.S, N669.S, N672.S, N676.S, N681.S, N687, N689, N690.S, N692.S, N693, N695.S, N698.S, N703.S, N709, N712.S, N714.S, N730, N734.S, N737.S, N738.S, N769, N775.S, N777.S, N779, N780.S, N781, N785, N788, N792, N793.S, N794, N797.S, N798.S, N802, N804, N807, N817.S, N827.S, N828, N830.S, N832.S, N833.S, N836.S, N840.S, N841, N844, N845, N849, N852, N858, N859, N860, N869.S, N871.S, N874.S, N875, N878, N880, N881, N885, N894, N898.S, N907.S, N913.S, N925, N927, N942, N947, N961, N968.S, N972, N982, N983, N986.S, N987, N988, N996, and N998.S.

4. The method of claim 1, wherein the network ecology consists essentially of N1008, N1023, N1051, N1061, N1070.S, N1088, N1089, N1092, N381.S, N382.S, N387.S, N399.S, N400.S, N402.S, N403.S, N414.S, N429.S, N430.S, N432.S, N433.S, N436.S, N437.S, N439.S, N441.S, N447.S, N448.S, N457.S, N460.S, N462.S, N463.S, N464.S, N470.S, N474.S, N488.S, N490.S, N493.S, N508.S, N509.S, N510.S, N511.S, N512.S, N514.S, N515.S, N517.S, N518.S, N519.S, N520.S, N523.S, N524.S, N529.S, N539.S, N543.S, N546.S, N547.S, N548.S, N570.S, N574.S, N577.S, N579.S, N580.S, N582.S, N584.S, N585.S, N589.S, N591.S, N597.S, N602.S, N604.S, N609.S, N610.S, N611.S, N612.S,

N614.S, N616.S, N621.S, N623.S, N625.S, N648.S, N651.S, N652.S, N653.S, N654.S, N655.S, N660.S, N663.S, N664, N665.S, N666.S, N669.S, N672.S, N676.S, N681.S, N687, N689, N690.S, N692.S, N693, N695.S, N698.S, N703.S, N709, N712.S, N714.S, N730, N734.S, N737.S, N738.S, N769, N775.S, N777.S, N779, N780.S, N781, N785, N788, N792, N793.S, N794, N797.S, N798.S, N802, N804, N807, N817.S, N827.S, N828, N830.S, N832.S, N833.S, N836.S, N840.S, N841, N844, N845, N849, N852, N858, N859, N860, N869.S, N871.S, N874.S, N875, N878, N880, N881, N885, N894, N898.S, N907.S, N913.S, N925, N927, N942, N947, N961, N968.S, N972, N982, N983, N986.S, N987, N988, N996, or N998.S.

5. The method of claim 3, wherein the network ecology is selected from the group consisting of N387.S, N399.S, N512.S, N462.S, N651.S, N982, and N845.

6. The method of claim 5, wherein the network ecology comprises N387.S and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_262, clade_396, clade_444, clade_478, clade_500, and clade_553.

7. The method of claim 5, wherein the network ecology comprises N387.S and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_262, clade_396, clade_444, clade_478, clade_500, and clade_553.

8. The method of claim 6 or claim 7, wherein clade_262 comprises one or more bacteria selected from the group consisting Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, and Ruminococcus torques, wherein clade_396 comprises one or more bacteria selected from the group consisting Acetivibrio ethanolgignens, Anaerosporobacter mobilis, Bacteroides pectinophilus, Clostridium aminovalericum, Clostridium phytofermentans, Eubacterium hallii, and Eubacterium xylanophilum, wherein clade_444 comprises one or more bacteria selected from the group consisting Butyrivibrio fibrisolvens, Eubacterium rectale, Eubacterium sp. oral clone GI038, Lachnobacterium bovis, Roseburia cecicola, Roseburia faecalis, Roseburia faecis, Roseburia hominis, Roseburia intestinalis, Roseburia inulinivorans, Roseburia sp. 11SE37, Roseburia sp. 11SE38, Shuttleworthia satelles, Shuttleworthia sp. MSX8B, and Shuttleworthia sp. oral taxon G69, wherein clade_478 comprises one or more bacteria selected from the group consisting Faecalibacterium prausnitzii, Gemmiger formicilis, and Subdoligranulum variabile, wherein clade_500 comprises one or more bacteria selected from the group consisting Alistipes finegoldii, Alistipes onderdonkii, Alistipes putredinis, Alistipes shahii, Alistipes sp. HGB5, Alistipes sp.

JC50, and Alistipes sp. RMA 9912, and wherein clade_553 comprises one or more bacteria selected from the group consisting Collinsella aerofaciens, Collinsella intestinalis, Collinsella stercoris, and Collinsella tanakaei.

9. The method of claim 6 or claim 7, wherein clade_262 comprises one or more bacteria of Ruminococcus torques, wherein clade_396 comprises one or more bacteria of Eubacterium hallii, wherein clade_444 comprises one or more bacteria selected from the group consisting of Eubacterium rectale and Roseburia inulinivorans, wherein clade_478 comprises one or more bacteria of Faecalibacterium prausnitzii, wherein clade_500 comprises one or more bacteria of Alistipes putredinis, and wherein clade_553 comprises one or more bacteria of Collinsella aerofaciens.

10. The method of claim 6 or claim 7, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_396 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 161, Seq. ID No.: 288, Seq. ID No.: 551, Seq. ID No.: 6, Seq. ID No.: 613, Seq. ID No.: 848, and Seq. ID No.: 875, wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865, wherein clade_478 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1896, Seq. ID No.: 880, and Seq. ID No.: 932, wherein clade_500 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 129, Seq. ID No.: 131, Seq. ID No.: 132, Seq. ID No.: 133, Seq. ID No.: 134, Seq. ID No.: 135, and Seq. ID No.: 136, and wherein clade_553 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 659, Seq. ID No.: 660, Seq. ID No.: 661, and Seq. ID No.: 662.

11. The method of claim 6 or claim 7, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_396 comprises one more bacteria

selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 161, Seq. ID No.: 288, Seq. ID No.: 551, Seq. ID No.: 6, Seq. ID No.: 613, Seq. ID No.: 848, and Seq. ID No.: 875, wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865, wherein clade_478 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1896, Seq. ID No.: 880, and Seq. ID No.: 932, wherein clade_500 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 129, Seq. ID No.: 131, Seq. ID No.: 132, Seq. ID No.: 133, Seq. ID No.: 134, Seq. ID No.: 135, and Seq. ID No.: 136, and wherein clade_553 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 659, Seq. ID No.: 660, Seq. ID No.: 661, and Seq. ID No.: 662.

12. The method of claim 6 or claim 7, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1670, wherein clade_396 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 848, wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1639 and Seq. ID No.: 856, wherein clade_478 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 880, wherein clade_500 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 132, and wherein clade_553 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 659.

13. The method of claim 6 or claim 7, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1670, wherein clade_396 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 848, wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1639 and Seq. ID No.: 856, wherein clade_478 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 880, wherein clade_500

comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 132, and wherein clade_553 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 659.

14. The method of claim 5, wherein the network ecology comprises N399.S and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_262, clade_360, clade_396, clade_444, clade_478, and clade_494.

15. The method of claim 5, wherein the network ecology comprises N399.S and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_262, clade_360, clade_396, clade_444, clade_478, and clade_494.

16. The method of claim 14 or claim 15, wherein clade_262 comprises one or more bacteria selected from the group consisting of Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, and Ruminococcus torques, wherein clade_360 comprises one or more bacteria selected from the group consisting of Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus, and Ruminococcus sp. ID8, wherein clade_396 comprises one or more bacteria selected from the group consisting of Acetivibrio ethanolgignens, Anaerosporobacter mobilis, Bacteroides pectinophilus, Clostridium aminovalericum, Clostridium phytofermentans, Eubacterium hallii, and Eubacterium xylanophilum, wherein clade_444 comprises one or more bacteria selected from the group consisting of Butyrivibrio fibrisolvens, Eubacterium rectale, Eubacterium sp. oral clone GI038, Lachnobacterium bovis, Roseburia cecicola, Roseburia faecalis, Roseburia faecis, Roseburia hominis, Roseburia intestinalis, Roseburia inulinivorans, Roseburia sp. 11SE37, Roseburia sp. 11SE38, Shuttleworthia satelles, Shuttleworthia sp. MSX8B, and Shuttleworthia sp. oral taxon G69, wherein clade_478 comprises one or more bacteria selected from the group consisting of Faecalibacterium prausnitzii, Gemmiger formicilis, and Subdoligranulum variabile, and wherein clade_494 comprises one or more bacteria selected from the group consisting of Clostridium orbiscindens, Clostridium sp. NML 04A032, Flavonifractor plautii, Pseudoflavonifractor capillosus, and Ruminococcaceae bacterium D16.

17. The method of claim 14 or 15, wherein clade_262 comprises one or more bacteria of Ruminococcus torques, wherein clade_360 comprises one or more bacteria of Dorea longicatena, wherein clade_396 comprises one or more bacteria of Eubacterium hallii,

wherein clade_444 comprises one or more bacteria of Eubacterium rectale, wherein clade_478 comprises one or more bacteria of Faecalibacterium prausnitzii, and wherein clade_494 comprises one or more bacteria of Pseudoflavonifractor capillosus.

18. The method of claim 14 or 15, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, wherein clade_396 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 161, Seq. ID No.: 288, Seq. ID No.: 551, Seq. ID No.: 6, Seq. ID No.: 613, Seq. ID No.: 848, and Seq. ID No.: 875, wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865, wherein clade_478 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1896, Seq. ID No.: 880, and Seq. ID No.: 932, and wherein clade_494 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1591, Seq. ID No.: 1655, Seq. ID No.: 609, Seq. ID No.: 637, and Seq. ID No.: 886.

19. The method of claim 14 or 15, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, wherein clade_396 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 161, Seq. ID No.: 288, Seq. ID No.: 551, Seq. ID No.: 6, Seq. ID No.: 613, Seq. ID No.: 848, and Seq. ID No.: 875, wherein clade_444 comprises one more bacteria selected from the group

consisting of bacteria having 16S sequences Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865, wherein clade_478 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1896, Seq. ID No.: 880, and Seq. ID No.: 932, and wherein clade_494 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1591, Seq. ID No.: 1655, Seq. ID No.: 609, Seq. ID No.: 637, and Seq. ID No.: 886.

20. The method of claim 14 or 15, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1670, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 774, wherein clade_396 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 848, wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 856, wherein clade_478 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 880, and wherein clade_494 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1591.

21. The method of claim 14 or 15, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1670, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 774, wherein clade_396 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 848, wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 856, wherein clade_478 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 880, and wherein clade_494 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1591.

22. The method of claim 5, wherein the network ecology comprises N462.S and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_262, clade_360, and clade_478.

23. The method of claim 5, wherein the network ecology comprises N462.S and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_262, clade_360, and clade_478.

24. The method of claim 22 or claim 23, wherein clade_262 comprises one or more bacteria selected from the group consisting of Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, and Ruminococcus torques, wherein clade_360 comprises one or more bacteria selected from the group consisting of Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus, and Ruminococcus sp. ID8, and wherein clade_478 comprises one or more bacteria selected from the group consisting of Faecalibacterium prausnitzii, Gemmiger formicilis, and Subdoligranulum variabile.

25. The method of claim 22 or claim 23, wherein clade_262 comprises one or more bacteria of Coprococcus comes, wherein clade_360 comprises one or more bacteria of Dorea longicatena, and wherein clade_478 comprises one or more bacteria selected from the group consisting Faecalibacterium prausnitzii and Subdoligranulum variabile.

26. The method of claim 22 or claim 23, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, and wherein clade_478 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1896, Seq. ID No.: 880, and Seq. ID No.: 932.

27. The method of claim 22 or claim 23, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668,

Seq. ID No.: 773, and Seq. ID No.: 774, and wherein clade_478 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1896, Seq. ID No.: 880, and Seq. ID No.: 932.

28. The method of claim 22 or claim 23, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 674, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 774, and wherein clade_478 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1896 and Seq. ID No.: 880.

29. The method of claim 22 or claim 23, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 674, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 774, and wherein clade_478 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1896 and Seq. ID No.: 880.

30. The method of claim 5, wherein the network ecology comprises N512.S and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_262, clade_360, and clade_444.

31. The method of claim 5, wherein the network ecology comprises N512.S and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_262, clade_360, and clade_444.

32. The method of claim 30 or claim 31, wherein clade_262 comprises one or more bacteria selected from the group consisting of Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, and Ruminococcus torques, wherein clade_360 comprises one or more bacteria selected from the group consisting of Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus, and Ruminococcus sp. ID8, and wherein clade_444 comprises one or more bacteria selected from the group consisting of Butyrivibrio fibrisolvens, Eubacterium rectale, Eubacterium sp. oral clone GI038, Lachnobacterium bovis, Roseburia cecicola, Roseburia faecalis, Roseburia faecis, Roseburia hominis, Roseburia intestinalis, Roseburia inulinivorans, Roseburia sp.

11SE37, Roseburia sp. 11SE38, Shuttleworthia satelles, Shuttleworthia sp. MSX8B, and Shuttleworthia sp. oral taxon G69.

33. The method of claim 30 or claim 31, wherein clade_262 comprises one or more bacteria selected from the group consisting of Coprococcus comes and Ruminococcus torques, wherein clade_360 comprises one or more bacteria of Dorea longicatena, and wherein clade_444 comprises one or more bacteria of Eubacterium rectale.

34. The method of claim 30 or claim 31, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, and wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865.

35. The method of claim 30 or claim 31, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, and wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865.

36. The method of claim 30 or claim 31, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1670 and Seq. ID No.: 674, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having

97% or greater identity to Seq. ID No.: 774, and wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 856.

37. The method of claim 30 or claim 31, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1670 and Seq. ID No.: 674, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 774, and wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 856.

38. The method of claim 5, wherein the network ecology comprises N845 and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_262, clade_360, and clade_378.

39. The method of claim 5, wherein the network ecology comprises N845 and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_262, clade_360, and clade_378.

40. The method of claim 38 or claim 39, wherein clade_262 comprises one or more bacteria selected from the group consisting of Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, and Ruminococcus torques, wherein clade_360 comprises one or more bacteria selected from the group consisting of Dorea formicigenerans, Dorea longicatena, Lachnospiraceae bacterium 2_1_46FAA, Lachnospiraceae bacterium 2_1_58FAA, Lachnospiraceae bacterium 4_1_37FAA, Lachnospiraceae bacterium 9_1_43BFAA, Ruminococcus gnavus, and Ruminococcus sp. ID8, and wherein clade_378 comprises one or more bacteria selected from the group consisting of Bacteroides barnesiae, Bacteroides coprocola, Bacteroides coprophilus, Bacteroides dorei, Bacteroides massiliensis, Bacteroides plebeius, Bacteroides sp. 3_1_33FAA, Bacteroides sp. 3_1_40A, Bacteroides sp. 4_3_47FAA, Bacteroides sp. 9_1_42FAA, Bacteroides sp. NB_8, and Bacteroides vulgatus.

41. The method of claim 38 or claim 39, wherein clade_262 comprises one or more bacteria of Coprococcus comes, wherein clade_360 comprises one or more bacteria of Dorea longicatena, and wherein clade_378 comprises one or more bacteria of Bacteroides dorei.

42. The method of claim 38 or claim 39, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq.

ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, and wherein clade_378 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 267, Seq. ID No.: 272, Seq. ID No.: 273, Seq. ID No.: 274, Seq. ID No.: 284, Seq. ID No.: 289, Seq. ID No.: 309, Seq. ID No.: 310, Seq. ID No.: 313, Seq. ID No.: 314, Seq. ID No.: 323, and Seq. ID No.: 331.

43. The method of claim 38 or claim 39, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, wherein clade_360 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1050, Seq. ID No.: 1051, Seq. ID No.: 1053, Seq. ID No.: 1058, Seq. ID No.: 1661, Seq. ID No.: 1668, Seq. ID No.: 773, and Seq. ID No.: 774, and wherein clade_378 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 267, Seq. ID No.: 272, Seq. ID No.: 273, Seq. ID No.: 274, Seq. ID No.: 284, Seq. ID No.: 289, Seq. ID No.: 309, Seq. ID No.: 310, Seq. ID No.: 313, Seq. ID No.: 314, Seq. ID No.: 323, and Seq. ID No.: 331.

44. The method of claim 38 or claim 39, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 674, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 774, and wherein clade_378 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 274.

45. The method of claim 38 or claim 39, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 674, wherein clade_360 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 774, and wherein clade_378 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 274.

46. The method of claim 5, wherein the network ecology comprises N982 and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_172, clade_262, and clade_396.

47. The method of claim 5, wherein the network ecology comprises N982 and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_172, clade_262, and clade_396.

48. The method of claim 46 or claim 47, wherein clade_172 comprises one or more bacteria selected from the group consisting of Bifidobacteriaceae genomosp. C1, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium infantis, Bifidobacterium kashiwanohense, Bifidobacterium longum, Bifidobacterium pseudocatenulatum, Bifidobacterium pseudolongum, Bifidobacterium scardovii, Bifidobacterium sp. HM2, Bifidobacterium sp. HMLN12, Bifidobacterium sp. M45, Bifidobacterium sp. MSX5B, Bifidobacterium sp. TM_7, and Bifidobacterium thermophilum, wherein clade_262 comprises one or more bacteria selected from the group consisting of Clostridium glycyrrhizinilyticum, Clostridium nexile, Coprococcus comes, Lachnospiraceae bacterium 1_1_57FAA, Lachnospiraceae bacterium 1_4_56FAA, Lachnospiraceae bacterium 8_1_57FAA, Ruminococcus lactaris, and Ruminococcus torques, and wherein clade_396 comprises one or more bacteria selected from the group consisting of Acetivibrio ethanolgignens, Anaerosporobacter mobilis, Bacteroides pectinophilus, Clostridium aminovalericum, Clostridium phytofermentans, Eubacterium hallii, and Eubacterium xylanophilum.

49. The method of claim 46 or claim 47, wherein clade_172 comprises one or more bacteria of Bifidobacterium longum, wherein clade_262 comprises one or more bacteria of Coprococcus comes, and wherein clade_396 comprises one or more bacteria of Eubacterium hallii.

50. The method of claim 46 or claim 47, wherein clade_172 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 345, Seq. ID No.: 346, Seq. ID No.: 347, Seq. ID No.: 348, Seq. ID No.: 350, Seq. ID No.: 351, Seq. ID No.: 352, Seq. ID No.: 353, Seq. ID No.: 354, Seq. ID No.: 355, Seq. ID No.: 356, Seq. ID No.: 357, Seq. ID No.: 358, Seq. ID No.: 359, Seq. ID No.: 360, Seq. ID No.: 361, Seq. ID No.: 362, Seq. ID No.: 363, Seq. ID No.: 364, and Seq. ID No.: 365, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.:

1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, and wherein clade_396 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 161, Seq. ID No.: 288, Seq. ID No.: 551, Seq. ID No.: 6, Seq. ID No.: 613, Seq. ID No.: 848, and Seq. ID No.: 875.

51. The method of claim 46 or claim 47, wherein clade_172 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 345, Seq. ID No.: 346, Seq. ID No.: 347, Seq. ID No.: 348, Seq. ID No.: 350, Seq. ID No.: 351, Seq. ID No.: 352, Seq. ID No.: 353, Seq. ID No.: 354, Seq. ID No.: 355, Seq. ID No.: 356, Seq. ID No.: 357, Seq. ID No.: 358, Seq. ID No.: 359, Seq. ID No.: 360, Seq. ID No.: 361, Seq. ID No.: 362, Seq. ID No.: 363, Seq. ID No.: 364, and Seq. ID No.: 365, wherein clade_262 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1048, Seq. ID No.: 1049, Seq. ID No.: 1057, Seq. ID No.: 1663, Seq. ID No.: 1670, Seq. ID No.: 588, Seq. ID No.: 607, and Seq. ID No.: 674, and wherein clade_396 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 161, Seq. ID No.: 288, Seq. ID No.: 551, Seq. ID No.: 6, Seq. ID No.: 613, Seq. ID No.: 848, and Seq. ID No.: 875.

52. The method of claim 46 or claim 47, wherein clade_172 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 356, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 674, and wherein clade_396 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 848.

53. The method of claim 46 or claim 47, wherein clade_172 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 356, wherein clade_262 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 674, and wherein clade_396 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 848.

54. The method of claim 5, wherein the network ecology comprises N651.S and the therapeutic bacterial composition comprises at least one bacterium selected from each of clade_444, clade_516, and clade_522.

55. The method of claim 5, wherein the network ecology comprises N651.S and the therapeutic bacterial composition consists essentially of at least one bacterium selected from each of clade_444, clade_516, and clade_522.

56. The method of claim 54 or claim 55, wherein clade_444 comprises one or more bacteria selected from the group consisting of Butyrivibrio fibrisolvens, Eubacterium rectale, Eubacterium sp. oral clone GI038, Lachnobacterium bovis, Roseburia cecicola, Roseburia faecalis, Roseburia faecis, Roseburia hominis, Roseburia intestinalis, Roseburia inulinivorans, Roseburia sp. 11SE37, Roseburia sp. 11SE38, Shuttleworthia satelles, Shuttleworthia sp. MSX8B, and Shuttleworthia sp. oral taxon G69, wherein clade_516 comprises one or more bacteria selected from the group consisting of Anaerotruncus colihominis, Clostridium methylpentosum, Clostridium sp. YIT 12070, Hydrogenoanaerobacterium saccharovorans, Ruminococcus albus, and Ruminococcus flavefaciens, and wherein clade_522 comprises one or more bacteria selected from the group consisting of Bacteroides galacturonicus, Eubacterium eligens, Lachnospira multipara, Lachnospira pectinoschiza, and Lactobacillus rogosae.

57. The method of claim 54 or claim 55, wherein clade_444 comprises one or more bacteria of Roseburia inulinivorans, wherein clade_516 comprises one or more bacteria of Anaerotruncus colihominis, and wherein clade_522 comprises one or more bacteria of Eubacterium eligens.

58. The method of claim 54 or claim 55, wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.: 1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865, wherein clade_516 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1005, Seq. ID No.: 164, Seq. ID No.: 1656, Seq. ID No.: 1660, Seq. ID No.: 606, and Seq. ID No.: 642, and wherein clade_522 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1046, Seq. ID No.: 1047, Seq. ID No.: 1114, Seq. ID No.: 280, and Seq. ID No.: 845.

59. The method of claim 54 or claim 55, wherein clade_444 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1045, Seq. ID No.: 1634, Seq. ID No.: 1635, Seq. ID No.: 1636, Seq. ID No.: 1637, Seq. ID No.: 1638, Seq. ID No.: 1639, Seq. ID No.: 1640, Seq. ID No.: 1641, Seq. ID No.: 1728, Seq. ID No.:

1729, Seq. ID No.: 1730, Seq. ID No.: 456, Seq. ID No.: 856, and Seq. ID No.: 865, wherein clade_516 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1005, Seq. ID No.: 164, Seq. ID No.: 1656, Seq. ID No.: 1660, Seq. ID No.: 606, and Seq. ID No.: 642, and wherein clade_522 comprises one more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1046, Seq. ID No.: 1047, Seq. ID No.: 1114, Seq. ID No.: 280, and Seq. ID No.: 845.

60. The method of claim 54 or claim 55, wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 1639, wherein clade_516 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 164, and wherein clade_522 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences having 97% or greater identity to Seq. ID No.: 845.

61. The method of claim 54 or claim 55, wherein clade_444 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 1639, wherein clade_516 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 164, and wherein clade_522 comprises one or more bacteria selected from the group consisting of bacteria having 16S sequences Seq. ID No.: 845.

62. The method of any of claims 0-61 wherein said composition further comprises a pharmaceutically-acceptable excipient.

63. The method of claim 62, wherein the therapeutic bacterial composition is substantially depleted of a residual habitat product of a fecal material.

64. The method of any of claims 1-61, wherein said composition is formulated for oral administration.

65. The method of any of claims 1-63, wherein said composition is capable of inducing the formation of IgA, RegIII-gamma, IL-10, regulatory T cells, TGF-beta, alpha-defensin, beta-defensin, or an antimicrobial peptide in the mammalian subject.

66. The method of any of claims 1-61, wherein said composition is comestible.

67. A composition, comprising any of the compositions administered according to the methods of claims 1-61.

68. A dosage unit comprising predetermined ratios of the isolated bacteria present in the network ecology of any of claims 1-61.

69. A method for producing short chain fatty acids (SCFA) within a mammalian subject,

comprising: administering to said mammalian subject in need thereof an effective amount of a therapeutic bacterial composition, said therapeutic bacterial composition comprising a plurality of isolated bacteria or a purified bacterial preparation, the plurality of isolated bacteria of the purified bacterial preparation capable of forming one or a plurality of bacterial functional pathways, the one or plurality of bacterial functional pathways capable of forming a functional network ecology selected from the group consisting of N262.S, N290.S, N284.S, N271.S, N282.S, N288.S, N302.S, N279.S, N310.S, N323.S, N331.S, N332.S, N301.S, N312.S, N339.S, N325.S, N340.S, N341.S, N346.S, N338.S, N336.S, N345.S, N355.S, N356.S, N343.S, N329.S, N361.S, N353.S, N381.S, N344.S, N352.S, N357.S, N358.S, N369.S, N372.S, N375.S, N380.S, N374.S, N377.S, N368.S, N370.S, N373.S, N376.S, N389.S, N394.S, N431.S, N434.S, N390.S, N397.S, N387.S, N440.S, N396.S, N399.S, N403.S, N414.S, N430.S, N432.S, N436.S, N437.S, N457.S, N545, N386.S, N402.S, N405.S, N415.S, N421.S, N422.S, N423.S, N458.S, N459.S, N493.S, N416.S, N439.S, N447.S, N490.S, N526, N429.S, N433.S, N448.S, N488.S, N508.S, N509.S, N510.S, N511.S, N408.S, N446.S, N451.S, N474.S, N520.S, N521.S, N535.S, N516.S, N463.S, N518.S, N586, N450.S, N465.S, N519.S, N537.S, N419.S, N468.S, N477.S, N514.S, N382.S, N460.S, N462.S, N512.S, N517.S, N523.S, N547.S, N548.S, N577.S, N581.S, N585.S, N616.S, N466.S, N469.S, N480.S, N482.S, N484.S, N515.S, N533.S, N709, N730, N478.S, N572.S, N400.S, N543.S, N582.S, N621.S, N689, N769, N481.S, N525.S, N528.S, N534.S, N574.S, N580.S, N590.S, N591.S, N597.S, N664, N693, N530.S, N687, N470.S, N529.S, N539.S, N546.S, N570.S, N579.S, N602.S, N614.S, N648.S, N652.S, N655.S, N672.S, N681.S, N690.S, N692.S, N698.S, N737.S, N738.S, N785, N841, N878, N880, N881, N987, N988, N996, N1061, N479.S, N538.S, N542.S, N578.S, N609.S, N611.S, N617.S, N666.S, N675.S, N682.S, N844, N845, N846, N852, N876, N982, N1008, N649.S, N657.S, N678.S, N686.S, N710.S, N522.S, N651.S, N653.S, N654.S, N680.S, N712.S, N792, N802, N804, N807, N849, N858, N859, N875, N885, N942, N961, N972, N1051, N587.S, N589.S, N612.S, N625.S, N656.S, N714.S, N779, N781, N828, N829, N860, N894, N925, N927, N935, N947, N983, N1023, N441.S, N584.S, N794, N788, N524.S, N604.S, N610.S, N623.S, N663.S, N669.S, N676.S, N703.S, N775.S, N777.S, N780.S, N817.S, N827.S, N836.S, N871.S, N874.S, N898.S, N907.S, N998.S, N1088, N1089, N660.S, N665.S, N667.S, N733.S, N734.S, N739.S, N741.S, N782.S, N789.S, N796.S, N798.S, N800.S, N809.S, N816.S, N842.S, N843.S, N869.S, N986.S, N995.S, N1002.S, N1004.S, N1019.S, N1093, N668.S, N685.S, N835.S, N851.S, N464.S, N695.S, N776.S, N793.S, N815.S, N833.S, N891.S, N1070.S, N1092, N795.S, N797.S, N808.S, N811.S, N826.S,

N830.S, N832.S, N840.S, N945.S, N960.S, N968.S, N1091, N805.S, N822.S, N928.S, N936.S, N1078.S, and N913.S.

70. The method of claim 69, wherein the functional network ecology is selected from the group consisting of N1008, N1023, N1051, N1061, N1070.S, N1088, N1089, N1092, N381.S, N382.S, N399.S, N400.S, N402.S, N403.S, N414.S, N429.S, N430.S, N432.S, N433.S, N436.S, N437.S, N439.S, N441.S, N447.S, N448.S, N457.S, N460.S, N462.S, N463.S, N464.S, N470.S, N474.S, N488.S, N490.S, N493.S, N508.S, N509.S, N510.S, N511.S, N512.S, N514.S, N515.S, N517.S, N518.S, N519.S, N520.S, N523.S, N524.S, N528.S, N529.S, N539.S, N543.S, N546.S, N547.S, N548.S, N570.S, N574.S, N577.S, N579.S, N580.S, N582.S, N584.S, N585.S, N589.S, N591.S, N597.S, N602.S, N604.S, N609.S, N610.S, N611.S, N612.S, N614.S, N616.S, N621.S, N623.S, N625.S, N648.S, N651.S, N652.S, N653.S, N654.S, N655.S, N660.S, N663.S, N664, N665.S, N666.S, N669.S, N672.S, N676.S, N681.S, N687, N689, N690.S, N692.S, N693, N695.S, N698.S, N703.S, N709, N712.S, N714.S, N730, N734.S, N737.S, N738.S, N769, N775.S, N777.S, N779, N780.S, N781, N785, N788, N792, N793.S, N794, N797.S, N798.S, N802, N804, N807, N817.S, N827.S, N828, N830.S, N832.S, N833.S, N836.S, N840.S, N841, N844, N845, N849, N852, N858, N859, N860, N869.S, N871.S, N874.S, N875, N878, N880, N881, N885, N894, N898.S, N907.S, N913.S, N925, N927, N942, N947, N961, N968.S, N972, N982, N983, N986.S, N987, N988, N996, and N998.S.

71. The method of claim 70, wherein the functional network ecology is N528.S, and the plurality of bacterial functional pathways comprises the functional pathways of of KO:K00656, KO:K01069, KO:K01734, KO:K03417, KO:K03778, KO:K07246.

72. A method for catalyzing secondary metabolism of bile acids within a mammalian subject, comprising: administering to said mammalian subject in need thereof an effective amount of a therapeutic bacterial composition, said therapeutic bacterial composition comprising a plurality of isolated bacteria or a purified bacterial preparation, the plurality of isolated bacteria of the purified bacterial preparation capable of forming one or a plurality of bacterial functional pathways, the one or plurality of bacterial functional pathways capable of forming a functional network ecology selected from the group consisting of N262.S, N290.S, N284.S, N271.S, N282.S, N288.S, N302.S, N279.S, N310.S, N323.S, N331.S, N332.S, N301.S, N312.S, N339.S, N325.S, N340.S, N341.S, N346.S, N338.S, N336.S, N345.S, N355.S, N356.S, N343.S, N329.S, N361.S, N353.S, N381.S, N344.S, N352.S, N357.S, N358.S, N369.S, N372.S, N375.S, N380.S, N374.S, N377.S, N368.S, N370.S, N373.S, N376.S, N389.S, N394.S, N431.S, N434.S, N390.S, N397.S, N387.S, N440.S, N396.S,

N399.S, N403.S, N414.S, N430.S, N432.S, N436.S, N437.S, N457.S, N545, N386.S, N402.S, N405.S, N415.S, N421.S, N422.S, N423.S, N458.S, N459.S, N493.S, N416.S, N439.S, N447.S, N490.S, N526, N429.S, N433.S, N448.S, N488.S, N508.S, N509.S, N510.S, N511.S, N408.S, N446.S, N451.S, N474.S, N520.S, N521.S, N535.S, N516.S, N463.S, N518.S, N586, N450.S, N465.S, N519.S, N537.S, N419.S, N468.S, N477.S, N514.S, N382.S, N460.S, N462.S, N512.S, N517.S, N523.S, N547.S, N548.S, N577.S, N581.S, N585.S, N616.S, N466.S, N469.S, N480.S, N482.S, N484.S, N515.S, N533.S, N709, N730, N478.S, N572.S, N400.S, N543.S, N582.S, N621.S, N689, N769, N481.S, N525.S, N528.S, N534.S, N574.S, N580.S, N590.S, N591.S, N597.S, N664, N693, N530.S, N687, N470.S, N529.S, N539.S, N546.S, N570.S, N579.S, N602.S, N614.S, N648.S, N652.S, N655.S, N672.S, N681.S, N690.S, N692.S, N698.S, N737.S, N738.S, N785, N841, N878, N880, N881, N987, N988, N996, N1061, N479.S, N538.S, N542.S, N578.S, N609.S, N611.S, N617.S, N666.S, N675.S, N682.S, N844, N845, N846, N852, N876, N982, N1008, N649.S, N657.S, N678.S, N686.S, N710.S, N522.S, N651.S, N653.S, N654.S, N680.S, N712.S, N792, N802, N804, N807, N849, N858, N859, N875, N885, N942, N961, N972, N1051, N587.S, N589.S, N612.S, N625.S, N656.S, N714.S, N779, N781, N828, N829, N860, N894, N925, N927, N935, N947, N983, N1023, N441.S, N584.S, N794, N788, N524.S, N604.S, N610.S, N623.S, N663.S, N669.S, N676.S, N703.S, N775.S, N777.S, N780.S, N817.S, N827.S, N836.S, N871.S, N874.S, N898.S, N907.S, N998.S, N1088, N1089, N660.S, N665.S, N667.S, N733.S, N734.S, N739.S, N741.S, N782.S, N789.S, N796.S, N798.S, N800.S, N809.S, N816.S, N842.S, N843.S, N869.S, N986.S, N995.S, N1002.S, N1004.S, N1019.S, N1093, N668.S, N685.S, N835.S, N851.S, N464.S, N695.S, N776.S, N793.S, N815.S, N833.S, N891.S, N1070.S, N1092, N795.S, N797.S, N808.S, N811.S, N826.S, N830.S, N832.S, N840.S, N945.S, N960.S, N968.S, N1091, N805.S, N822.S, N928.S, N936.S, N1078.S, and N913.S.

73. The method of claim 72, wherein the functional network ecology is selected from the group consisting of N1008, N1023, N1051, N1061, N1070.S, N1088, N1089, N1092, N381.S, N382.S, N399.S, N400.S, N402.S, N403.S, N414.S, N429.S, N430.S, N432.S, N433.S, N436.S, N437.S, N439.S, N441.S, N447.S, N448.S, N457.S, N460.S, N462.S, N463.S, N464.S, N470.S, N474.S, N488.S, N490.S, N493.S, N508.S, N509.S, N510.S, N511.S, N512.S, N514.S, N515.S, N517.S, N518.S, N519.S, N520.S, N523.S, N524.S, N529.S, N539.S, N543.S, N546.S, N547.S, N548.S, N570.S, N574.S, N577.S, N579.S, N580.S, N582.S, N584.S, N585.S, N589.S, N591.S, N597.S, N602.S, N604.S, N609.S, N610.S, N611.S, N612.S, N614.S, N616.S, N621.S, N623.S, N625.S, N648.S, N651.S,

N652.S, N653.S, N654.S, N655.S, N660.S, N663.S, N664, N665.S, N666.S, N669.S, N672.S, N676.S, N681.S, N687, N689, N690.S, N692.S, N693, N695.S, N698.S, N703.S, N709, N712.S, N714.S, N730, N734.S, N737.S, N738.S, N769, N775.S, N777.S, N779, N780.S, N781, N785, N788, N792, N793.S, N794, N797.S, N798.S, N802, N804, N807, N817.S, N827.S, N828, N830.S, N832.S, N833.S, N836.S, N840.S, N841, N844, N845, N849, N852, N858, N859, N860, N869.S, N871.S, N874.S, N875, N878, N880, N881, N885, N894, N898.S, N907.S, N913.S, N925, N927, N942, N947, N961, N968.S, N972, N982, N983, N986.S, N987, N988, N996, and N998.S.

74. The method of claim 73, wherein the functional network ecology is N660.S and the plurality of bacterial functional pathways comprises the functional pathways of of KO:K00656, and KO:K01442.

75. The method of any of claims 69-74, wherein said composition further comprises a pharmaceutically-acceptable excipient.

76. The method of any of claims 69-74, wherein said composition is formulated for oral administration.

77. The method of any of claims 69-74, wherein said composition is capable of inducing the formation of butyrate, propionate, acetate, 7-deoxybile acids, deoxycholate acide (DCA) and lithocholic acid (LCA)in the mammalian subject.

78. The method of any of claims 69-74, wherein said composition is capable of inducing the depletion of glucose, pyruvate, lactate, cellulose, fructans, starch, xylans, pectins, taurocholate, glycocholate, ursocholate, cholate, glycochenodeoxycholate, taurochenodeoxycholate, ursodeoxycholate, or chenodeoxycholate; or the formation and depletion of intermediary metabolites acetyl-CoA, butyryl-CoA, propanoyl-CoA, chenodeoxycholoyl-CoA, or ursodeoxycholoyl-CoA in the mammalian subject.

79. The method of any of claims 69-74, wherein the composition is formulated with one or more prebiotic compounds.

80. The method of any of claims 69-74, wherein said composition is comestible.

81. A composition, comprising any of the compositions administered according to the methods of claims 69-74.

82. A dosage unit comprising predetermined ratios of the isolated bacteria present in the network ecology of any of claims 69-74.

83. A pharmaceutical formulation comprising a purified bacterial population consisting essentially of a bacterial network capable of forming germinable bacterial spores, wherein the bacterial network is present in an amount effective to populate the gastrointestinal tract in a

mammalian subject in need thereof to whom the formulation is administered, under conditions such that at least one type of bacteria not detectably present in the bacterial network or in the gastrointestinal tract prior to administration is augmented.

84. A pharmaceutical formulation comprising a purified bacterial population comprising a plurality of bacterial entities, wherein the bacterial entities are present in an amount effective to induce the formation of a functional bacterial network in the gastrointestinal tract in a mammalian subject in need thereof to whom the formulation is administered.

85. The formulation of claim 84, wherein the functional bacterial network comprises bacterial entities present in the formulation.

86. The formulation of claim 84, wherein the functional bacterial network comprises bacterial entities present in the gastrointestinal tract at the time of administration.

87. The formulation of claim 84, wherein the functional bacterial network comprises bacterial entities not present in the formulation or the gastrointestinal tract at the time of administration.

88. The formulation of claim 83 or claim 84, provided as an oral finished pharmaceutical dosage form including at least one pharmaceutically acceptable carrier.

89. The formulation of claim 83 or claim 84, wherein the mammalian subject suffers from a dysbiosis comprising a gastrointestinal disease, disorder or condition selected from the group consisting of *Clostridium difficile* Associated Diarrhea (CDAD), Type 2 Diabetes, Type 1 Diabetes, Obesity, Irritable Bowel Syndrome (IBS), Irritable Bowel Disease (IBD), Ulcerative Colitis, Crohn's Disease, colitis, colonization with a pathogen or pathobiont, and infection with a drug-resistant pathogen or pathobiont.

90. The formulation of claim 83, wherein the bacterial network is purified from a fecal material subjected to a treatment step that comprises depleting or inactivating a pathogenic material.

91. The formulation of claim 90, wherein the bacterial network is substantially depleted of a detectable level of a first pathogenic material.

92. The formulation of claim 90, wherein the bacterial network is substantially depleted of a residual habitat product of the fecal material.

93. A method of treating or preventing a dysbiosis in a human subject, comprising administering to the human subject the formulation of claim 83 or claim 84 in an amount effective to treat or prevent a dysbiosis or to reduce the severity of at least one symptom of the dysbiosis in the human subject to whom the formulation is administered.

94. The method of claim 93, wherein the formulation is provided as an oral finished

pharmaceutical dosage form including at least one pharmaceutically acceptable carrier, the dosage form comprising at least about $1x10^4$ colony forming units of bacterial spores per dose of the composition, wherein the bacterial spores comprise at least two bacterial entities comprising 16S rRNA sequences at least 97% identical to the nucleic acid sequences selected from the group consisting of Seq. ID No.: 674, Seq. ID No.: 1670, Seq. ID No.: 774, Seq. ID No.: 848, Seq. ID No.: 856, Seq. ID No.: 1639, Seq. ID No.: 880, Seq. ID No.: 1896, Seq. ID No.: 1591, Seq. ID No.: 164, Seq. ID No.: 845, and Seq. ID No.: 659.

95. The method of claim 93, wherein the administration of the formulation results in a reduction or an elimination of at least one pathogen and/or pathobiont present in the gastrointestinal tract when the therapeutic composition is administered.

96. The method of claim 93, wherein the administration of the formulation results in engraftment of at least one type of spore-forming bacteria present in the therapeutic composition.

97. The method of claim 93, wherein the administration of the formulation results in augmentation in the gastrointestinal tract of the subject to whom the formulation is administered of at least one type of bacteria not present in the formulation.

98. The method of claim 96, wherein the at least one type of spore-forming bacteria are not detectably present in the gastrointestinal tract of the subject to whom the formulation is administered when the formulation is administered.

99. The method of claim 93, wherein the administration of the formulation results in at least two of: i) reduction or elimination of at least one pathogen and/or pathobiont present in the gastrointestinal tract when the formulation is administered; ii) engraftment of at least one type of spore-forming bacteria present in the therapeutic composition; and iii) augmentation of at least one type of spore-forming or non-spore forming bacteria not present in the therapeutic composition.

100. The method of claim 90, wherein the administration of the therapeutic composition results in at reduction or elimination of at least one pathogen and/or pathobiont present in the gastrointestinal tract when the therapeutic composition is administered and at least one of: i) engraftment of at least one type of spore-forming bacteria present in the therapeutic composition; and ii) augmentation of at least one type of bacteria not present in the therapeutic composition.

101. A method of inducing engraftment of a bacterial population in the gastrointestinal tract of a human subject, comprising the step of administering to the human subject an orally acceptable pharmaceutical formulation comprising a purified bacterial network, under

conditions such that at least i) a subset of the spore-forming bacteria sustainably engraft within the gastrointestinal tract, or ii) at least one type of bacteria not present in the therapeutic composition is augmented within the gastrointestinal tract.

102. A pharmaceutical formulation comprising a purified first bacterial entity and a purified second bacterial entity, wherein the first bacterial entity comprises a first nucleic acid sequence encoding a first polypeptide capable of catalyzing a first chemical reaction, wherein the second bacterial entity comprises a second nucleic acid sequence encoding a second polypeptide capable of catalyzing a second chemical reaction, wherein the pharmaceutical formulation is formulated for oral administration to a mammalian subject in need thereof, wherein the first chemical reaction and the second chemical reaction are capable of occurring in the gastrointestinal tract of the mammalian subject under conditions such that a first product of the first chemical reaction, a substance present within said mammalian subject, or a combination of said first product with the substance is used as a substrate in the second chemical reaction to form a second product, wherein the second product induces a host cell response.

103. The formulation of claim 102, wherein said substance is a mammalian subject protein or a food-derived protein.

104. The pharmaceutical formulation of claim 102, wherein the host cell response comprises production by the host cell of a biological material.

105. The pharmaceutical formulation of claim 102, wherein the biological material comprises a cytokine, growth factor or signaling polypeptide.

106. The pharmaceutical formulation of claim 102, wherein the host cell response comprises an immune response.

107. The pharmaceutical formulation of claim 102, wherein the host cell response comprises decreased gastric motility.

108. The pharmaceutical formulation of claim 102, wherein the host cell response comprises change in host gene expression, increased host metabolism, reduced gut permeability, enhanced epithelial cell junction integrity, reduced lipolysis by the action of Lipoprotein Lipase in adipose tissue, decreased hepatic gluconeogenesis, increased insulin sensitivity, increased production of FGF-19, or change in energy harvesting and/or storage.

109. A pharmaceutical formulation comprising a purified first bacterial entity and a purified second bacterial entity, wherein the first bacterial entity and the second bacterial entity form a functional bacterial network in the gastrointestinal tract of a mammalian subject to whom the pharmaceutical formulation is administered, wherein the functional network

modulates the level and/or activity of a biological material capable of inducing a host cell response.

110. A pharmaceutical formulation comprising a purified first bacterial entity and a purified second bacterial entity, wherein the first bacterial entity and the second bacterial entity form a functional bacterial network in the gastrointestinal tract of a mammalian subject to whom the pharmaceutical formulation is administered, wherein the functional network induces the production of a biological material capable of inducing a host cell response.

111. A therapeutic composition, comprising a network of at least two bacterial entities, wherein the network comprises at least one keystone bacterial entity and at least one non-keystone bacterial entity, wherein the at least two bacterial entities are each provided in amounts effective for the treatment or prevention of a gastrointestinal disease, disorder or condition in a mammalian subject.

112. The composition of claim 111, wherein the network comprises at least three bacterial entities.

113. The composition of claim 111, wherein the network comprises at least three bacterial entities including at least two keystone bacterial entities.

114. A therapeutic composition, comprising a network of at least two keystone bacterial entities capable of forming germination-competent spores, wherein the at least two keystone bacterial entities are each provided in amounts effective for the treatment or prevention of a gastrointestinal disease, disorder or condition in a mammalian subject.

115. The therapeutic composition of claim 114, comprising a network of at least two keystone bacterial entities capable of forming germination-competent spores.

116. A therapeutic composition, comprising:

a first network of at least two bacterial entities, wherein the first network comprises a keystone bacterial entity and a non-keystone bacterial entity; and

a second network of at least two bacterial entities, wherein the second network comprises at least one keystone bacterial entity and at least one non-keystone bacterial entity,

wherein the networks are each provided in amounts effective for the treatment or prevention of a gastrointestinal disease, disorder or condition in a mammalian subject.

117. A therapeutic composition, comprising a network of at least two bacterial entities, wherein the network comprises a first keystone bacterial entity and a second keystone bacterial entity, wherein the two bacterial entities are each provided in amounts effective for

the treatment or prevention of a gastrointestinal disease, disorder or condition in a mammalian subject.

118. The composition of claim 117, wherein the first and second keystone bacterial entities are present in the same network.

119. The composition of claim 117, wherein the first and second keystone bacterial entities are present in different networks.

120. A diagnostic composition for the detection of a dysbiosis, comprising a first detection moiety capable of detecting a first keystone bacterial entity and a second detection moiety capable of detecting a first non-keystone bacterial entity, wherein the keystone bacterial entity and the non-keystone bacterial entity comprise a network, wherein the absence of at least one of the keystone bacterial entity and the non-keystone bacterial entity in a mammalian subject is indicative of a dysbiosis.

121. A method of altering a microbiome population present in a mammalian subject, comprising the steps of determining the presence of an incomplete network of bacterial entities in the gastrointestinal tract of the mammalian subject, and introducing to the gastrointestinal tract of the mammalian subject an effective amount of one or more supplemental bacterial entities not detectable in the gastrointestinal tract of the mammalian subject prior to such administration, under conditions such that the incomplete network is completed, thereby altering the microbiome population.

122. The method of claim 121, wherein the one or more supplemental bacterial entities become part of the incomplete network, thereby forming a complete network.

123. The method of claim 121, wherein the one or more supplemental bacterial entities alter the microbiota of the mammalian subject such that one or more additional bacterial entities complete the incomplete network.

124. The method of claim 121, where the one or more supplemental bacterial entities comprise a network.

125. A method for detection and correction of a dysbiosis in a mammalian subject in need thereof, comprising the steps of: providing a fecal sample from the mammalian subject comprising a plurality of bacterial entities; contacting the fecal sample with a first detection moiety capable of detecting a first bacterial entity present in an network; detecting the absence of the first bacterial entity in the fecal sample, thereby detecting a dysbiosis in the mammalian subject; and administering to the mammalian subject a composition comprising an effective amount of the first bacterial entity.

126. The method of claim 125, further comprising confirming that the dysbiosis in the

mammalian subject has been corrected.

127. A system for predicting a dysbiosis in a subject, the system comprising: a storage memory for storing a dataset associated with a sample obtained from the subject, wherein the dataset comprises content data for at least one network of bacterial entities; and a processor communicatively coupled to the storage memory for determining a score with an interpretation function wherein the score is predictive of dysbiosis in the subject.

128. A kit for diagnosis of a state of dysbiosis in a mammalian subject in need thereof, comprising a plurality of detection means suitable for use in detecting (1) a first bacterial entity comprising a keystone bacterial entity and (2) a second bacterial entity, wherein the first and second bacterial entities comprise a functional network ecology.

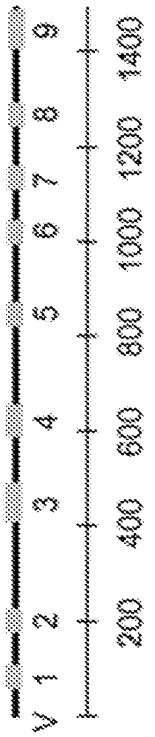

Figure 1

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151   ACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGG
201   GGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAG
251   TAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAG
301   GATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
351   CAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC
401   GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGA
451   AGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGC
501   ACCGGCTAACTCGTGCCCAGGCATGCGCAGGAATACGGAGGTGCAAGCGT
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601   ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGC
651   TTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT
701   AGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACT
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1001  GGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGC
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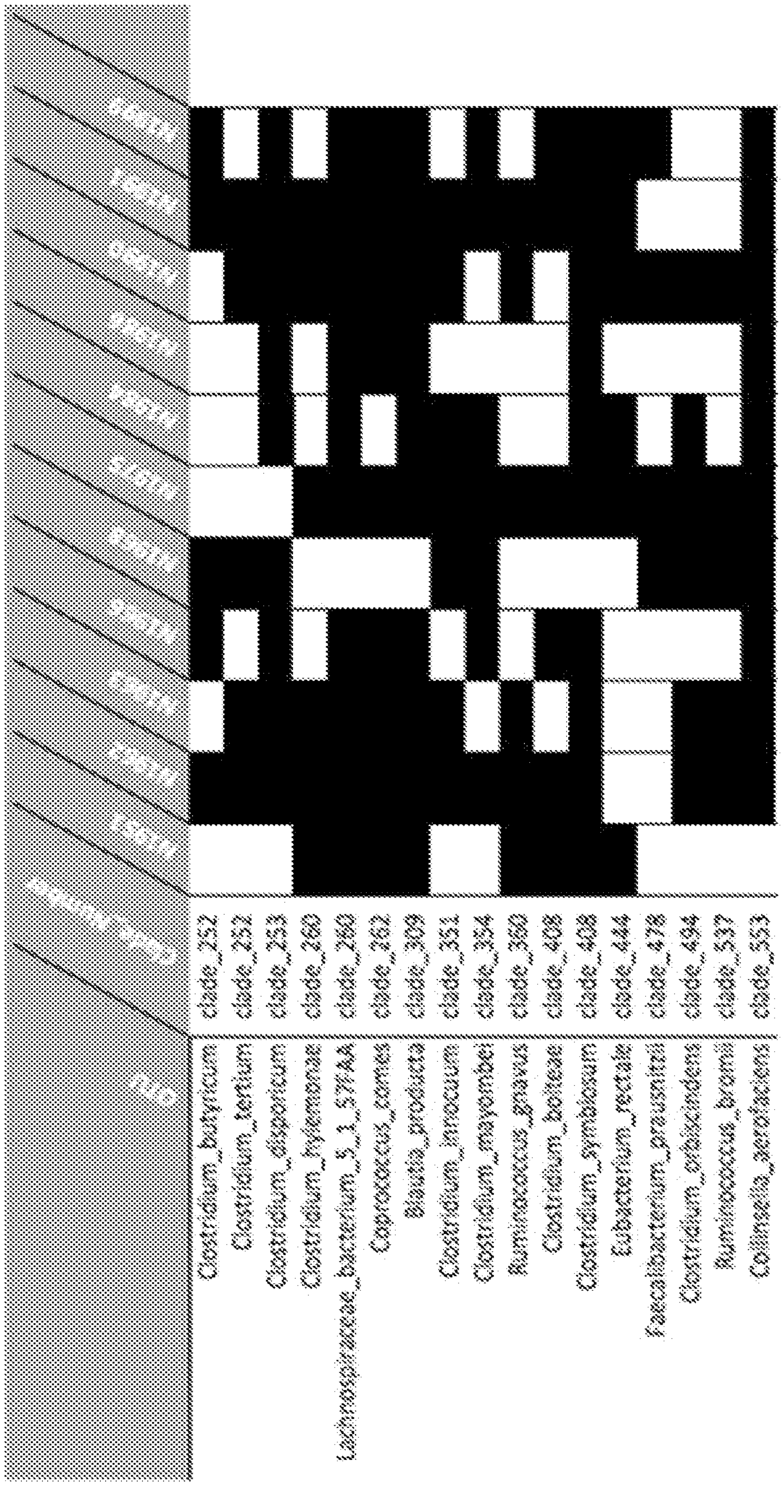
Figure 2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Clostridium_tertium clade_252
Clostridium_disporicum clade_253
Clostridium_hylemonae clade_260
Lachnospiraceae_bacterium_5_1_57FAA clade_260
Coprococcus_comes clade_262
Blautia_producta clade_309
Clostridium_innocuum clade_351
Clostridium_mayombei clade_354
Ruminococcus_gnavus clade_360
Clostridium_bolteae clade_408
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Eubacterium_rectale clade_444
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Collinsella_aerofaciens clade_553

Figure 3

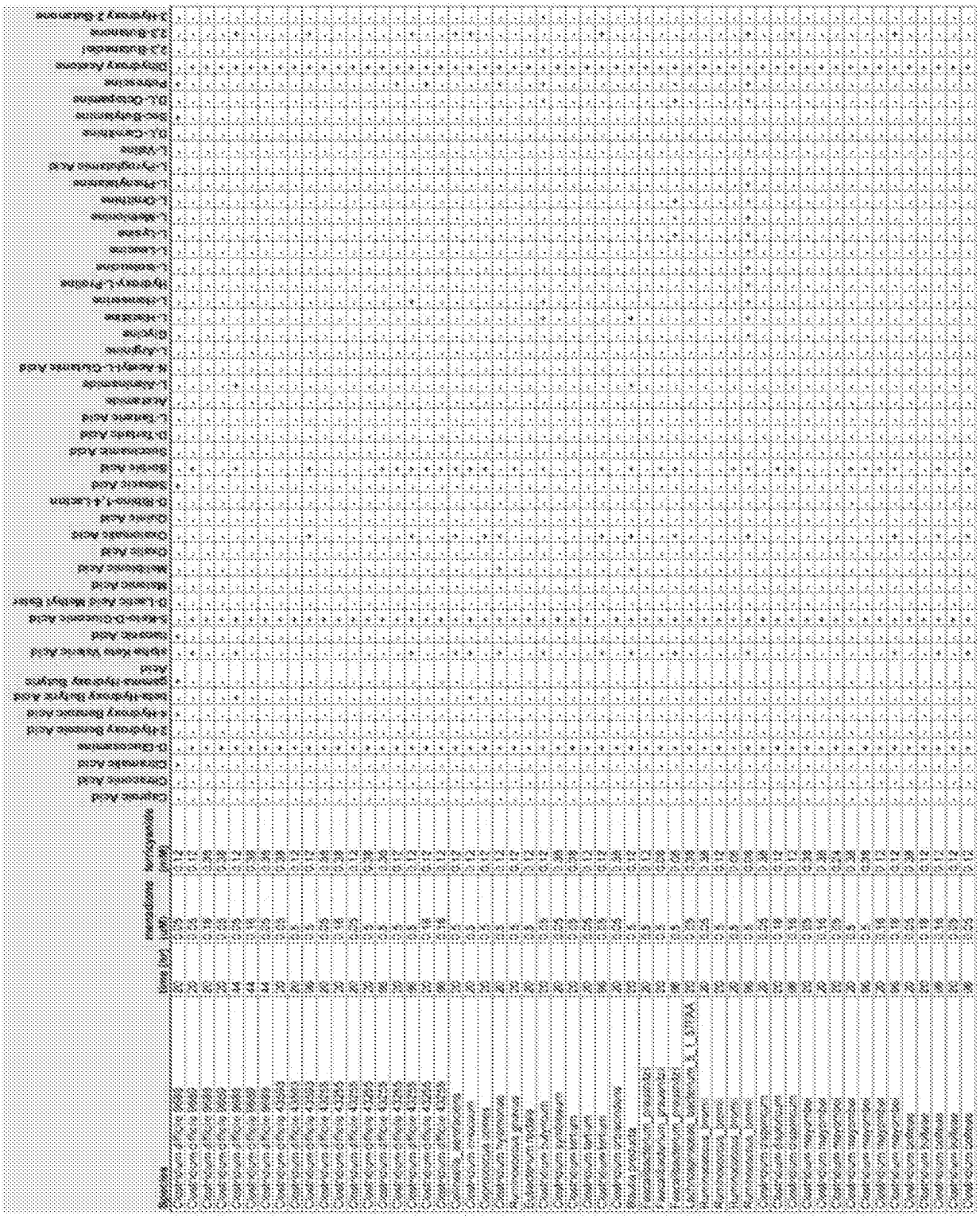


Figure 4

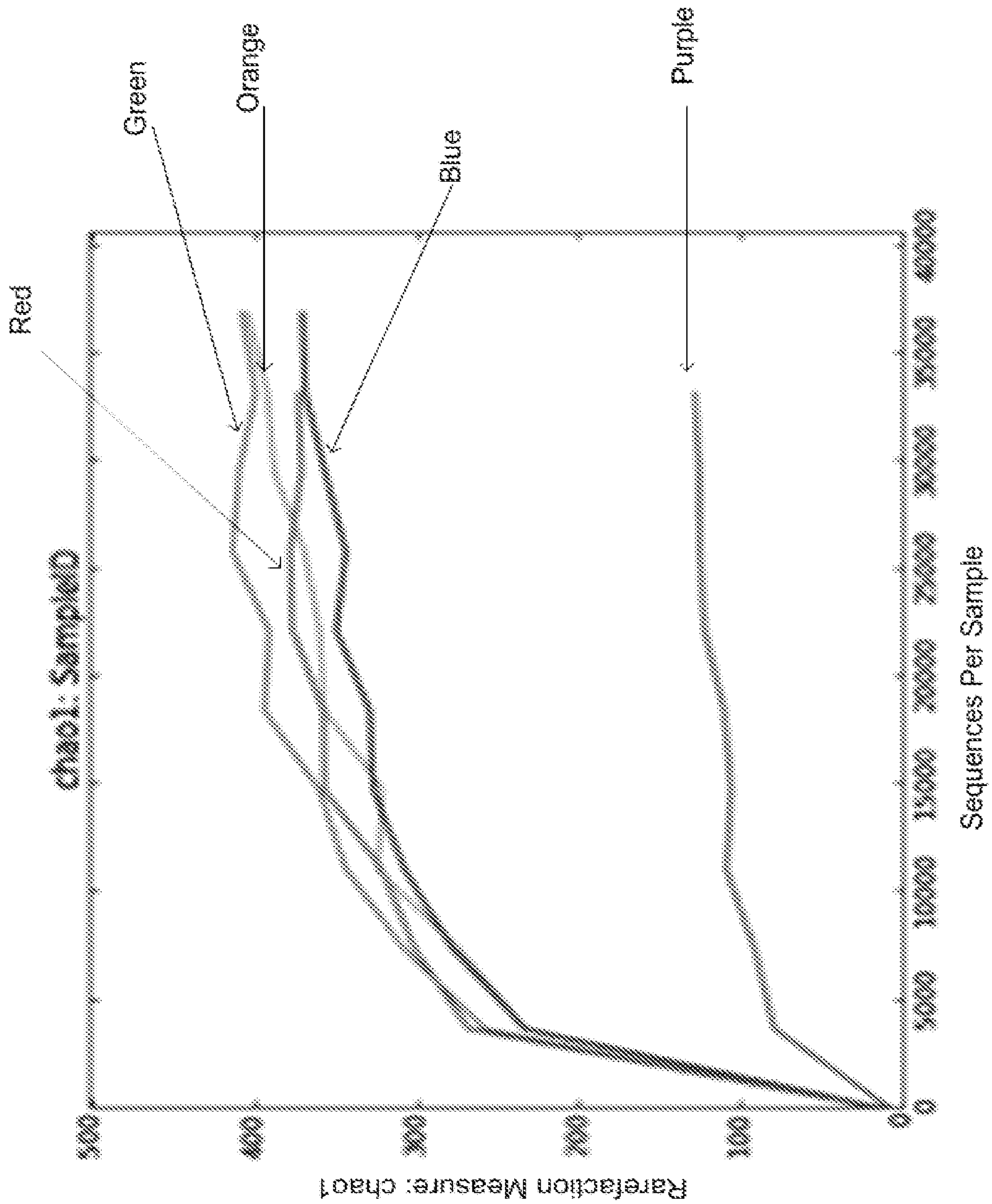

Red
Green
Orange
Blue
Purple
Sequences Per Sample
Rarefaction Measure: chao1

Figure 5

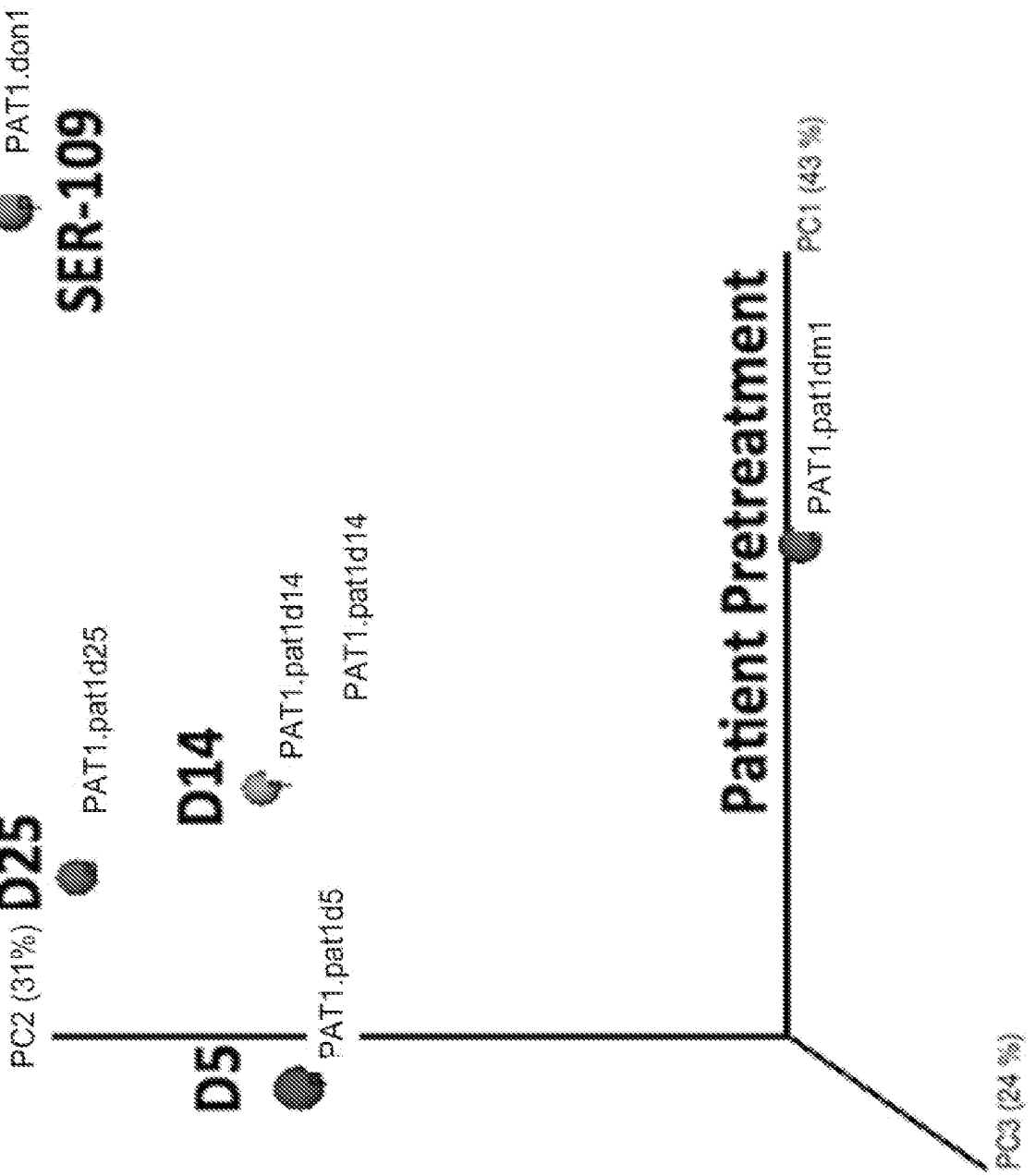
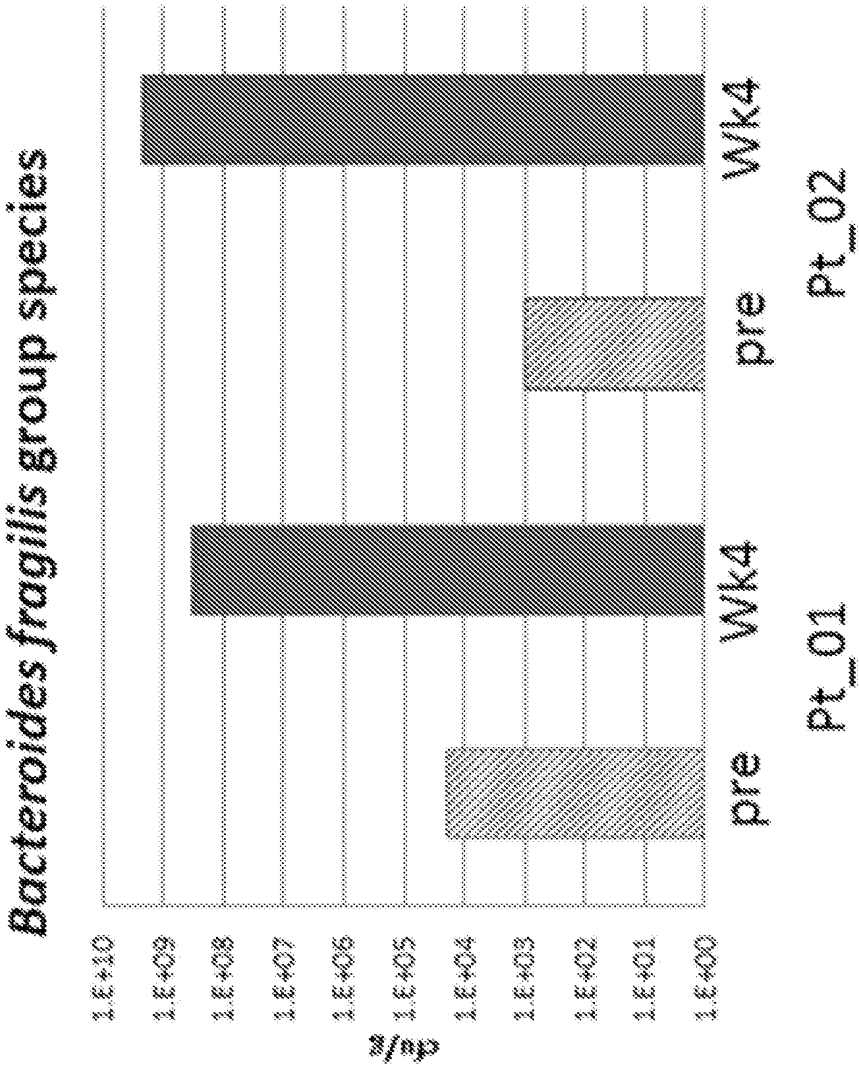


Figure 6


Bacteroides fragilis group species
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1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
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1.E+02
1.E+01
1.E+00
g/ap
pre
Wk4
Pt_01
pre
Wk4
Pt_02

Figure 7

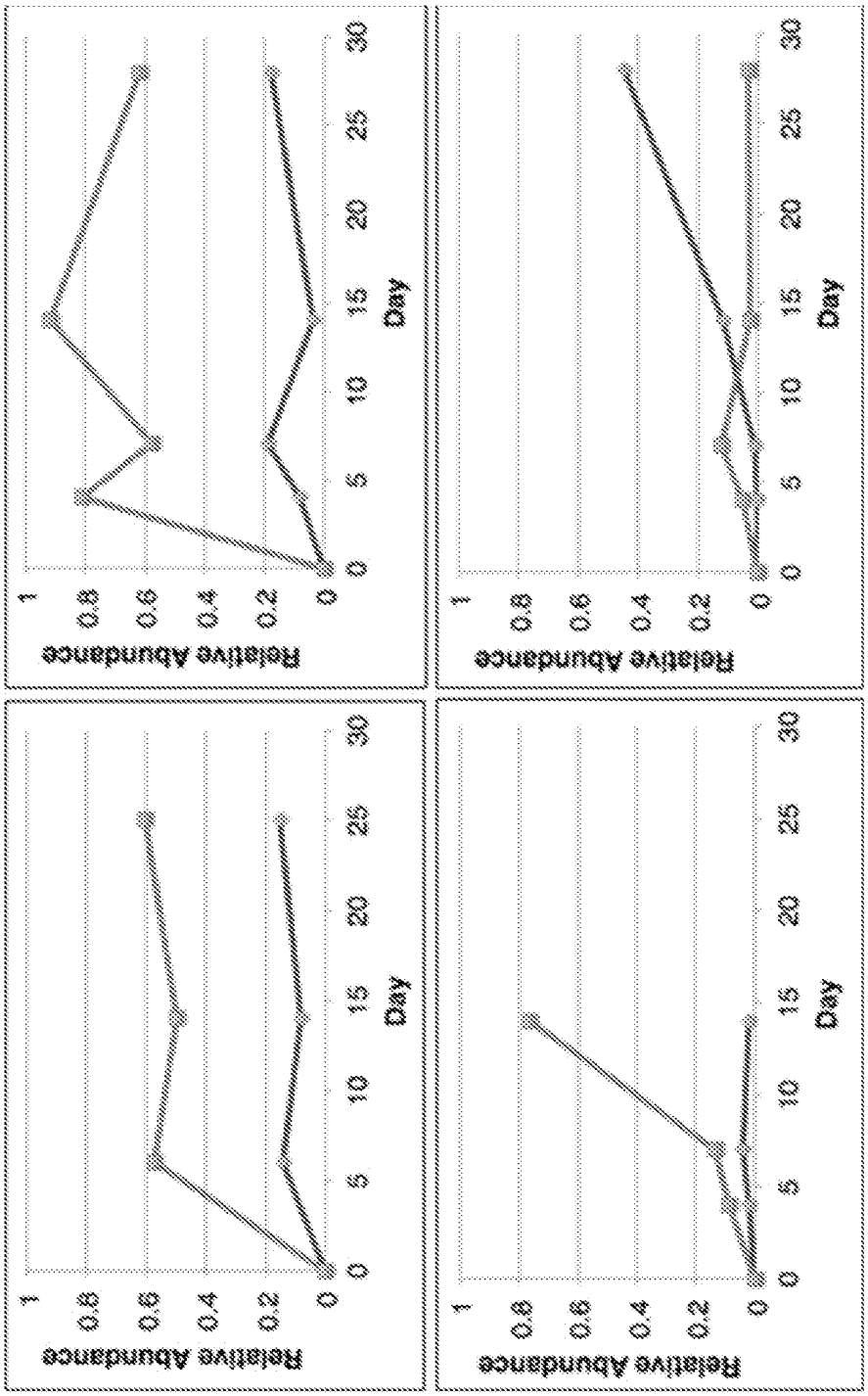


Figure 8

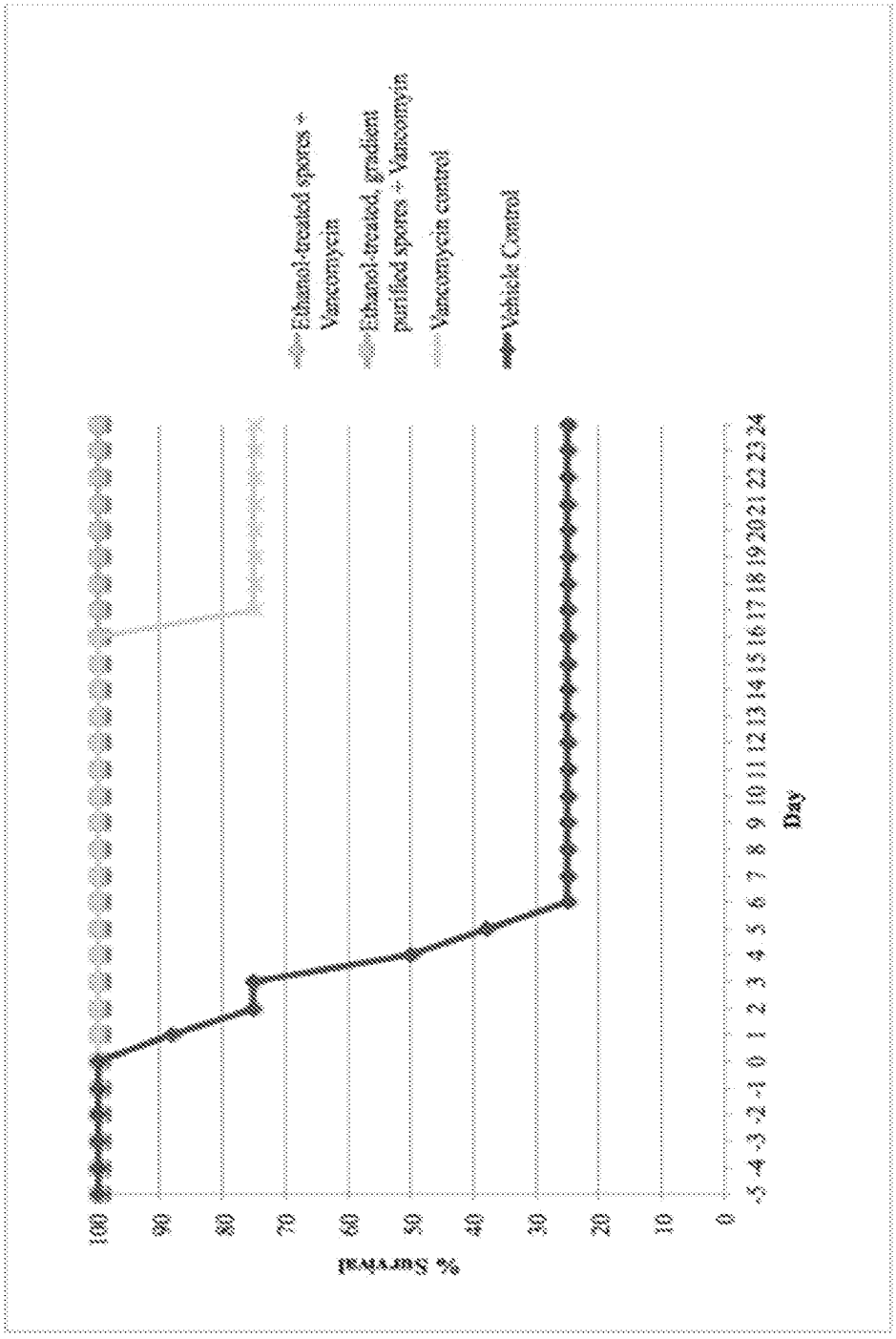
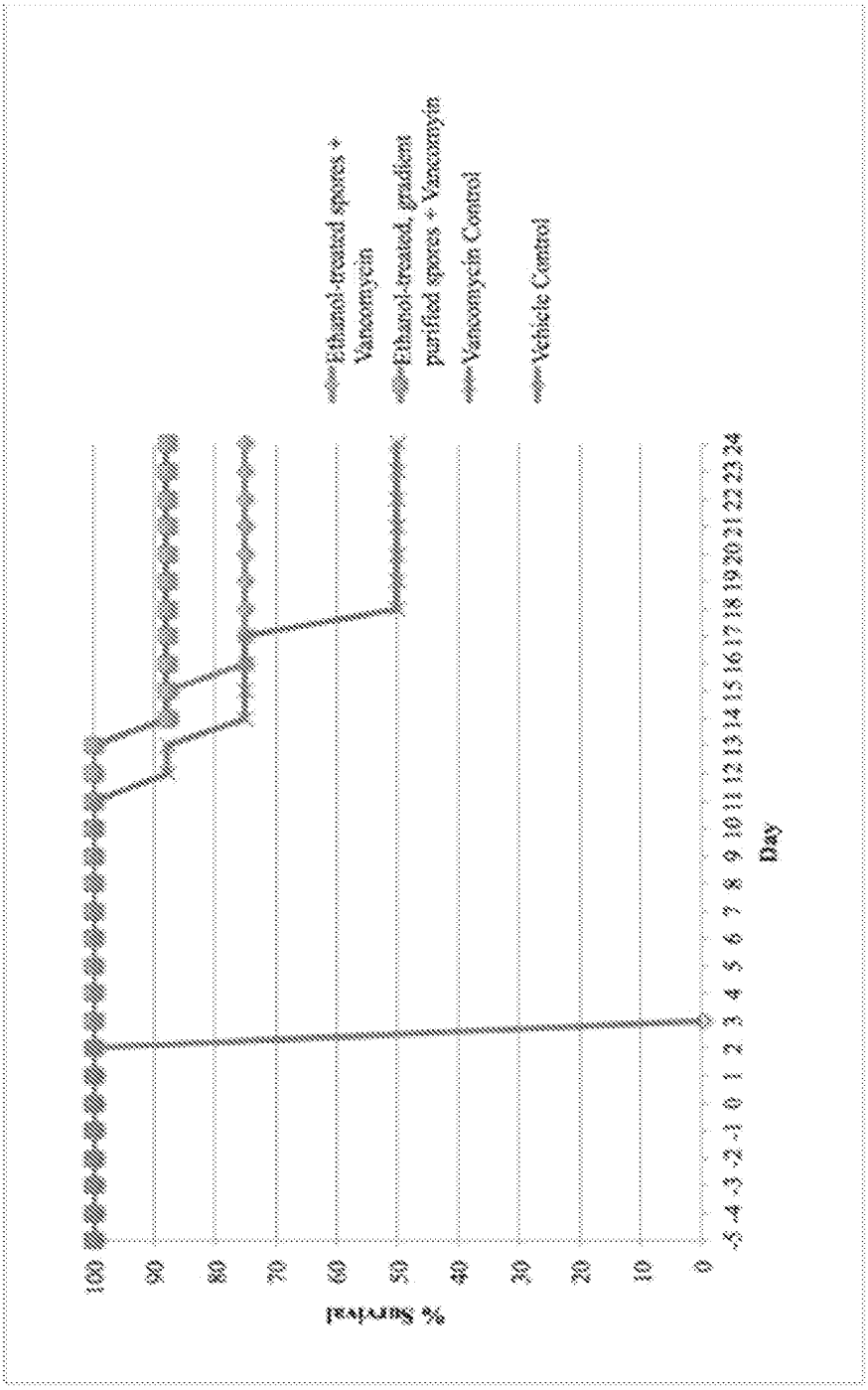

Ethanol-treated spores + Vancomycin
Ethanol-treated, gradient purified spores + Vancomycin
Vancomycin control
Vehicle Control
% Survival
Day

Figure 9


Ethanol-treated spores + Vancomycin
Ethanol-treated, gradient purified spores + Vancomycin
Vancomycin Control
Vehicle Control
% Survival
100
90
80
70
60
50
40
30
20
10
0
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Day

Figure 10

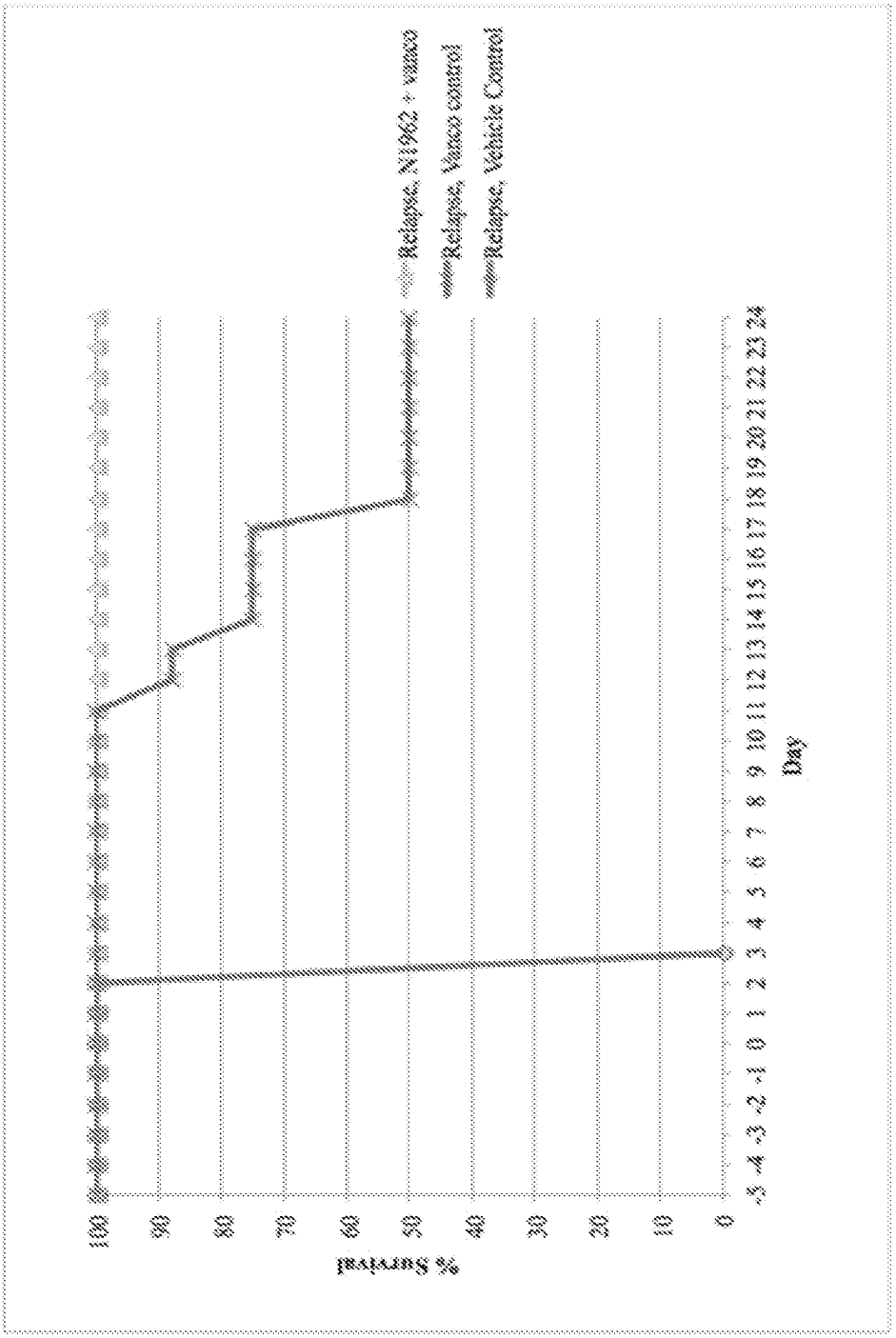


Figure 11

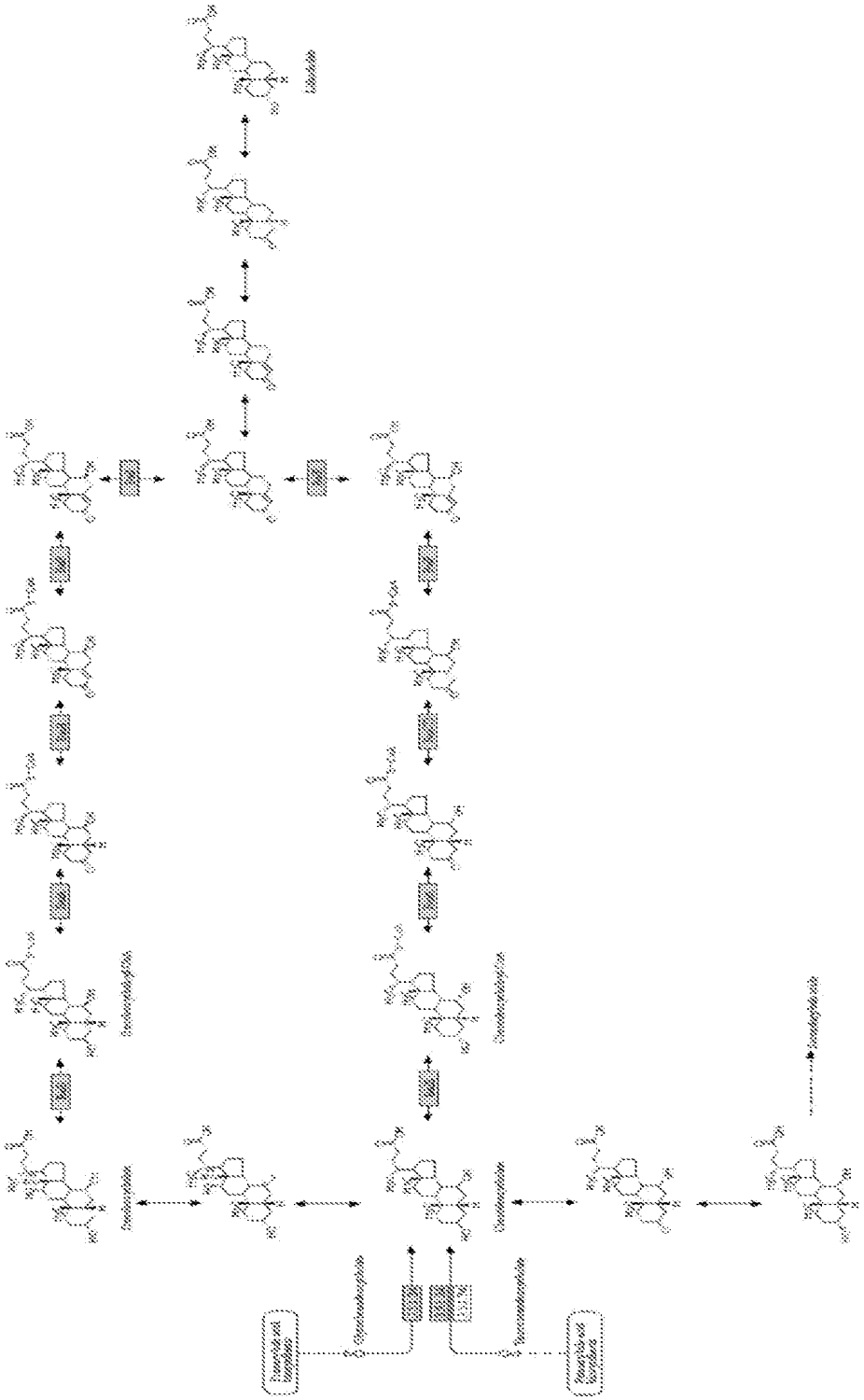


Figure 12

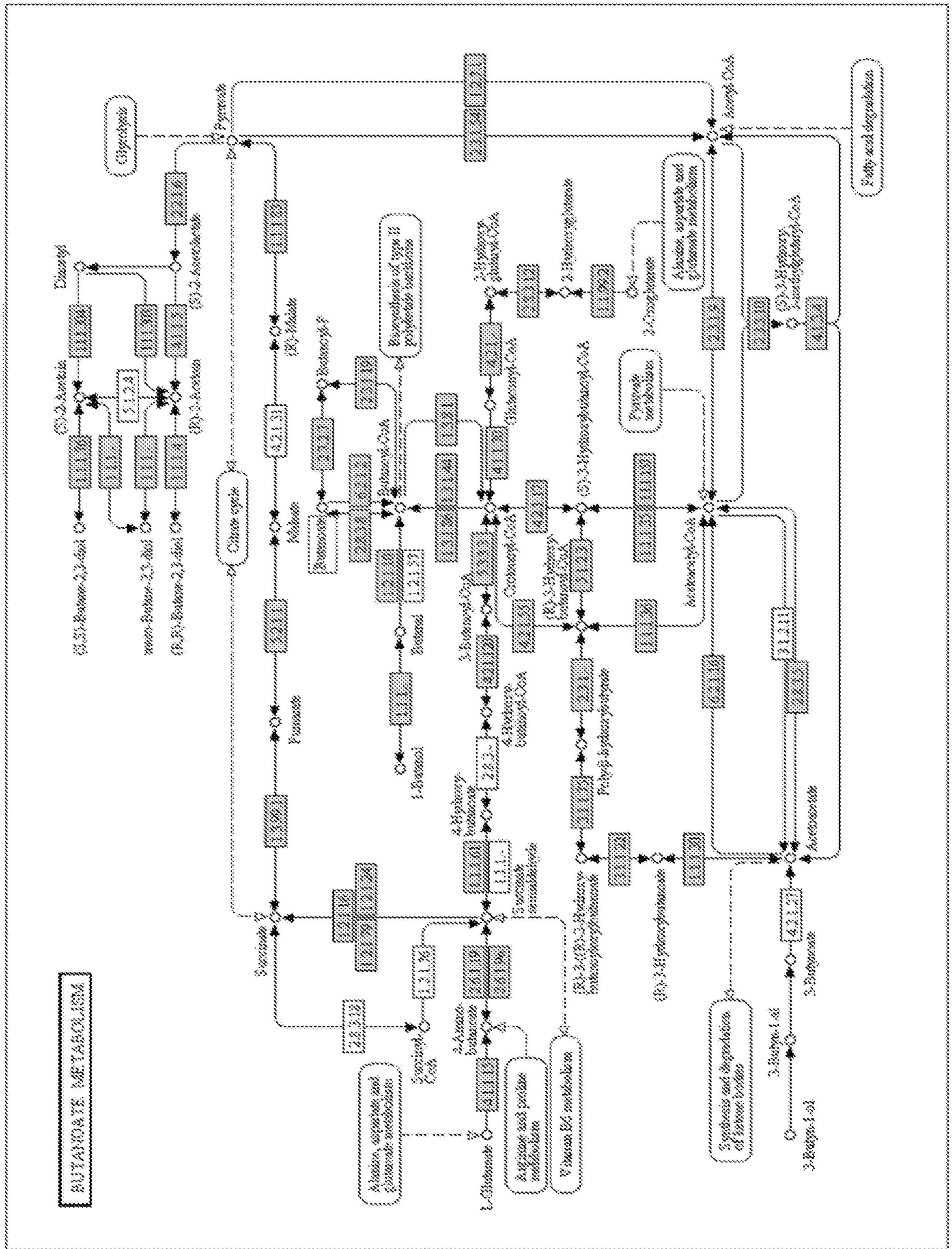


Figure 13

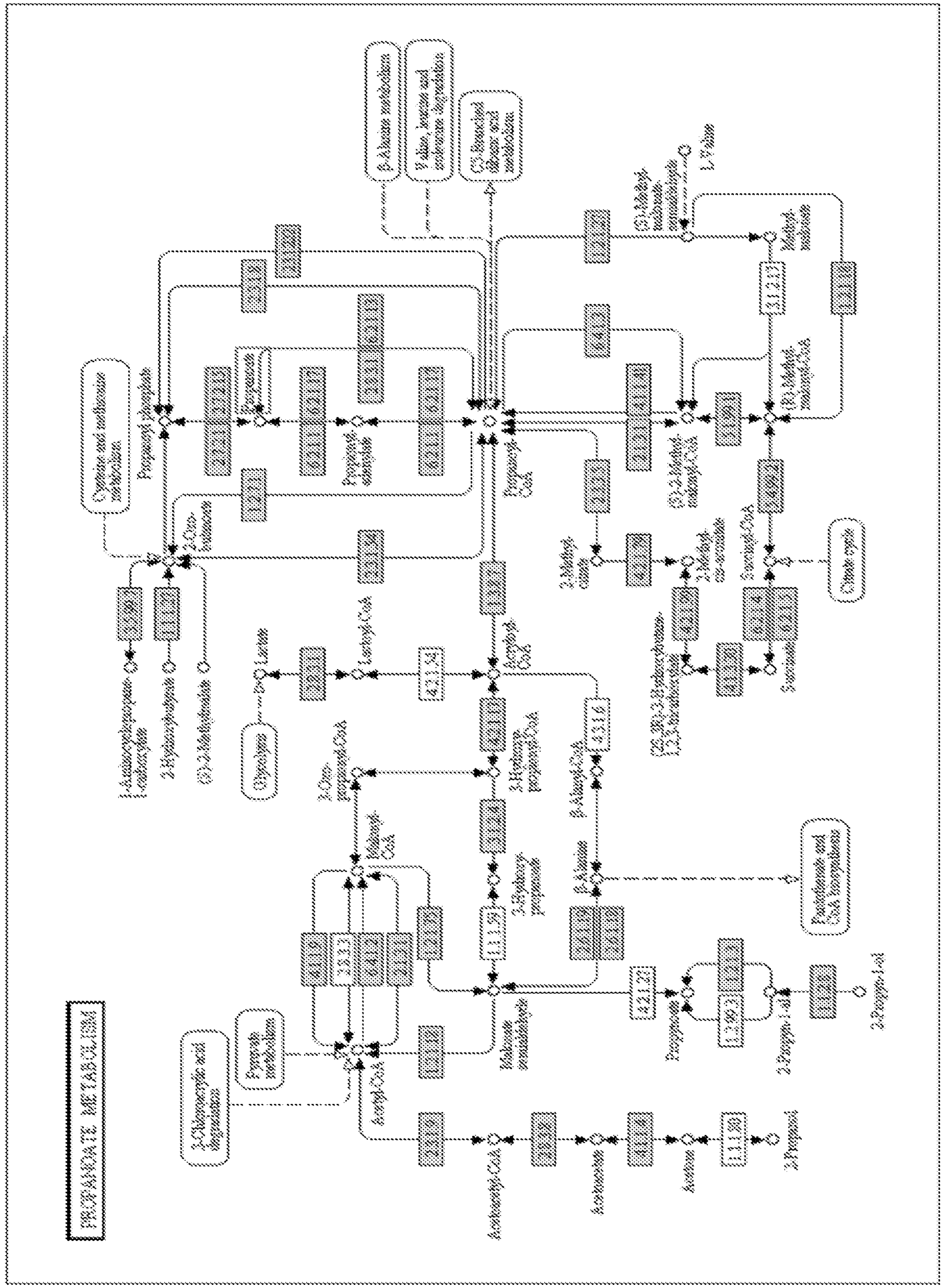


Figure 14

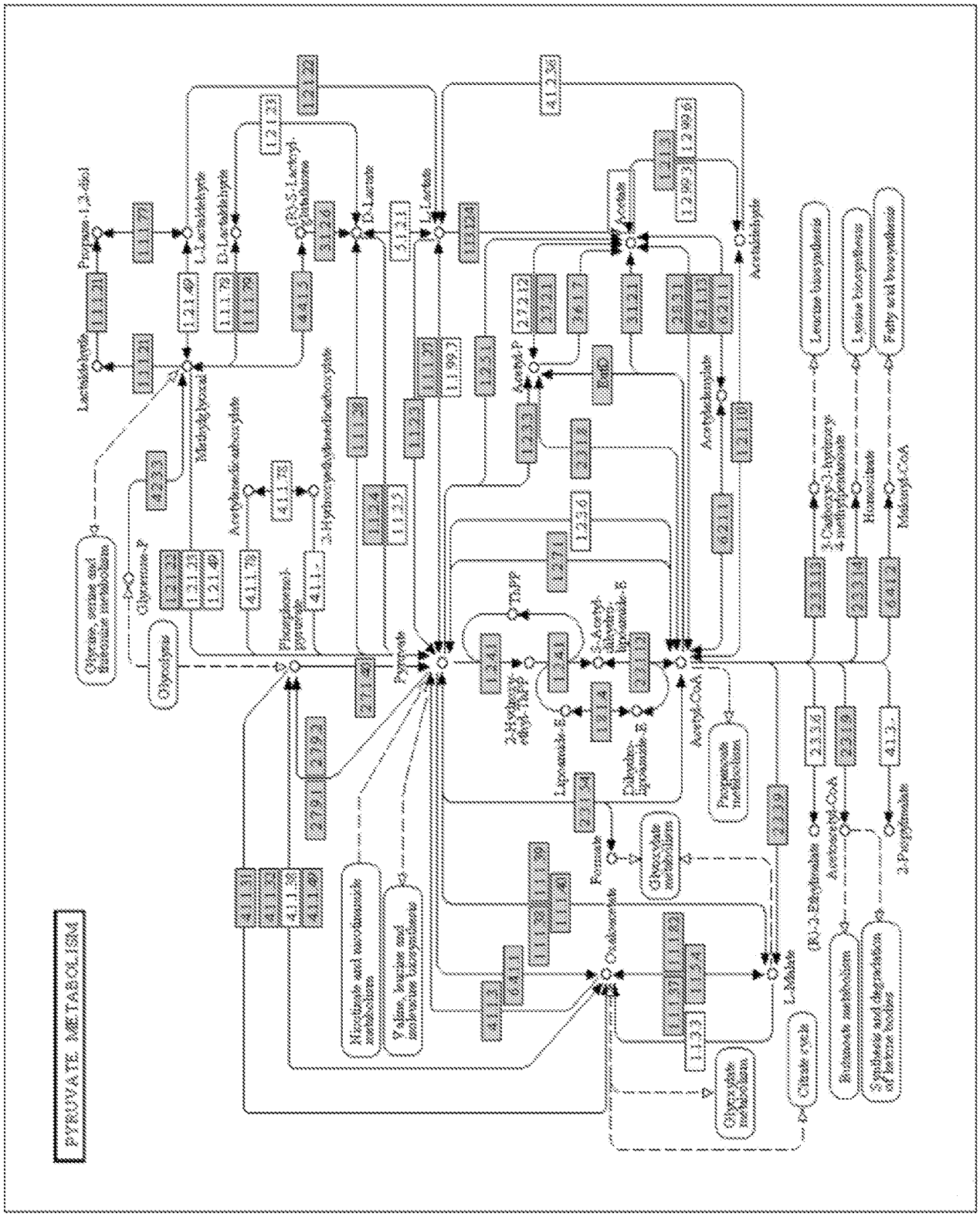
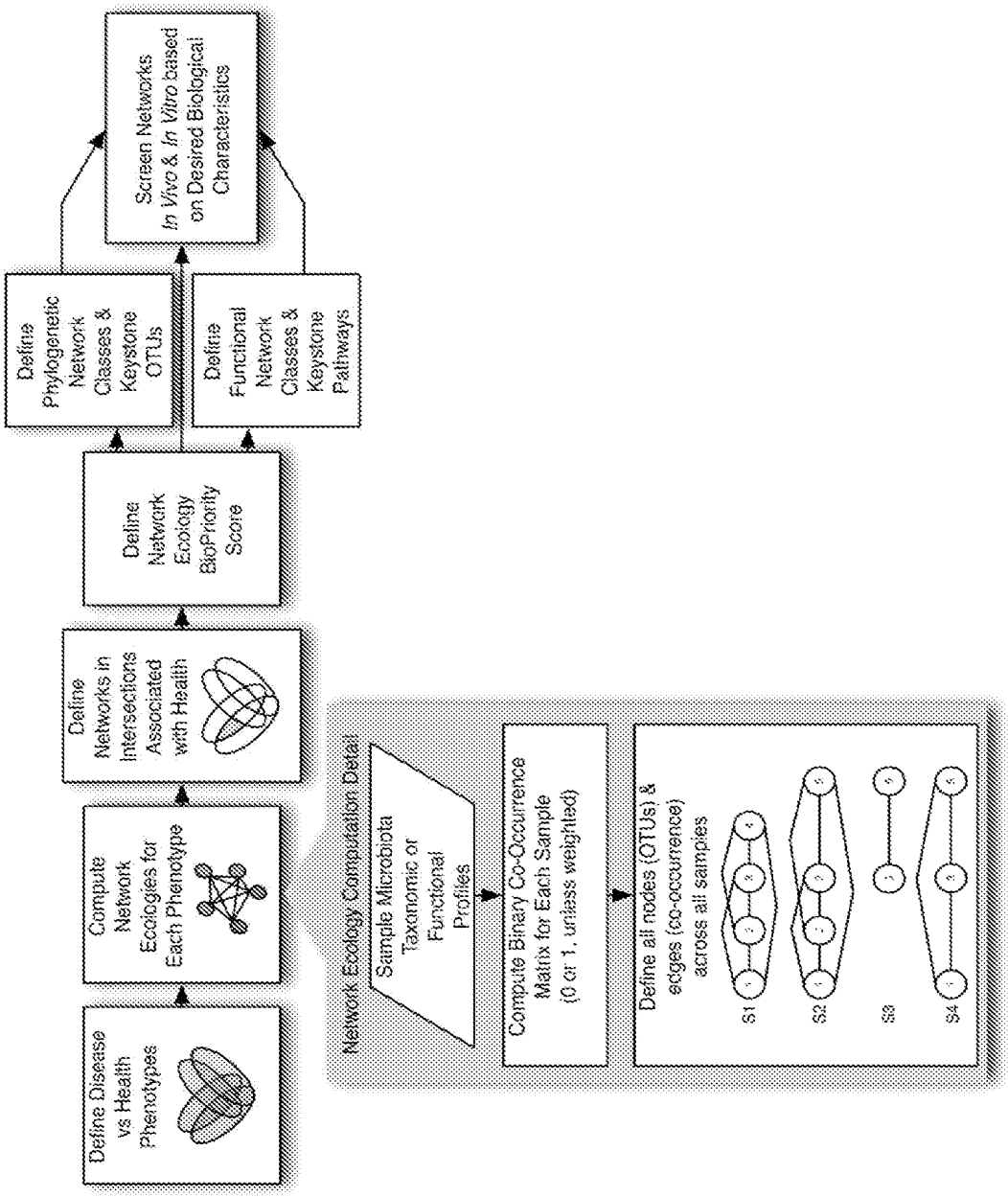


Figure 15



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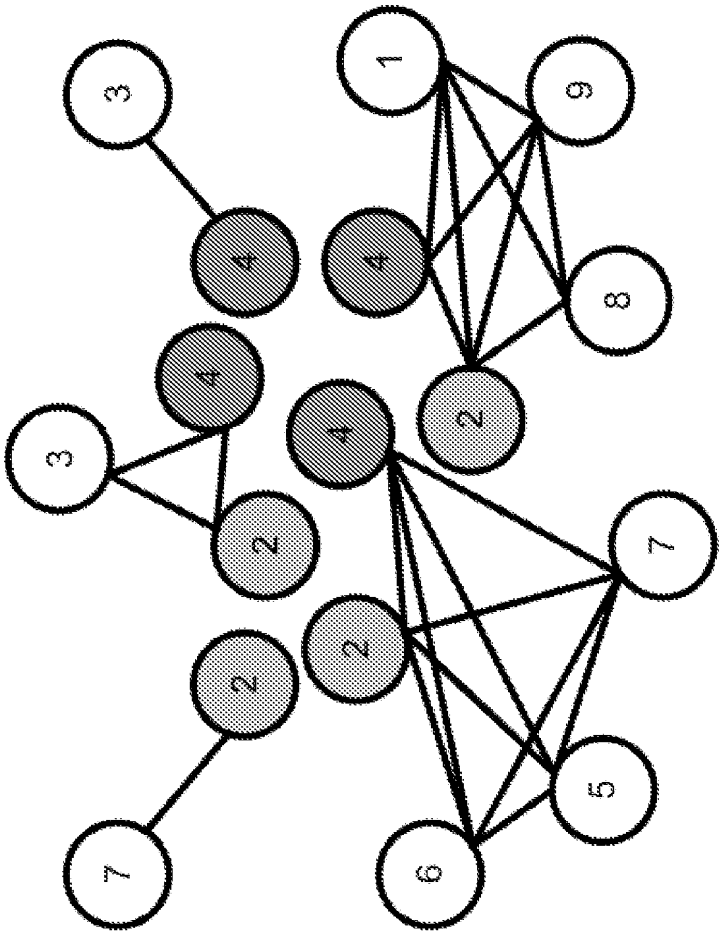


Figure 17

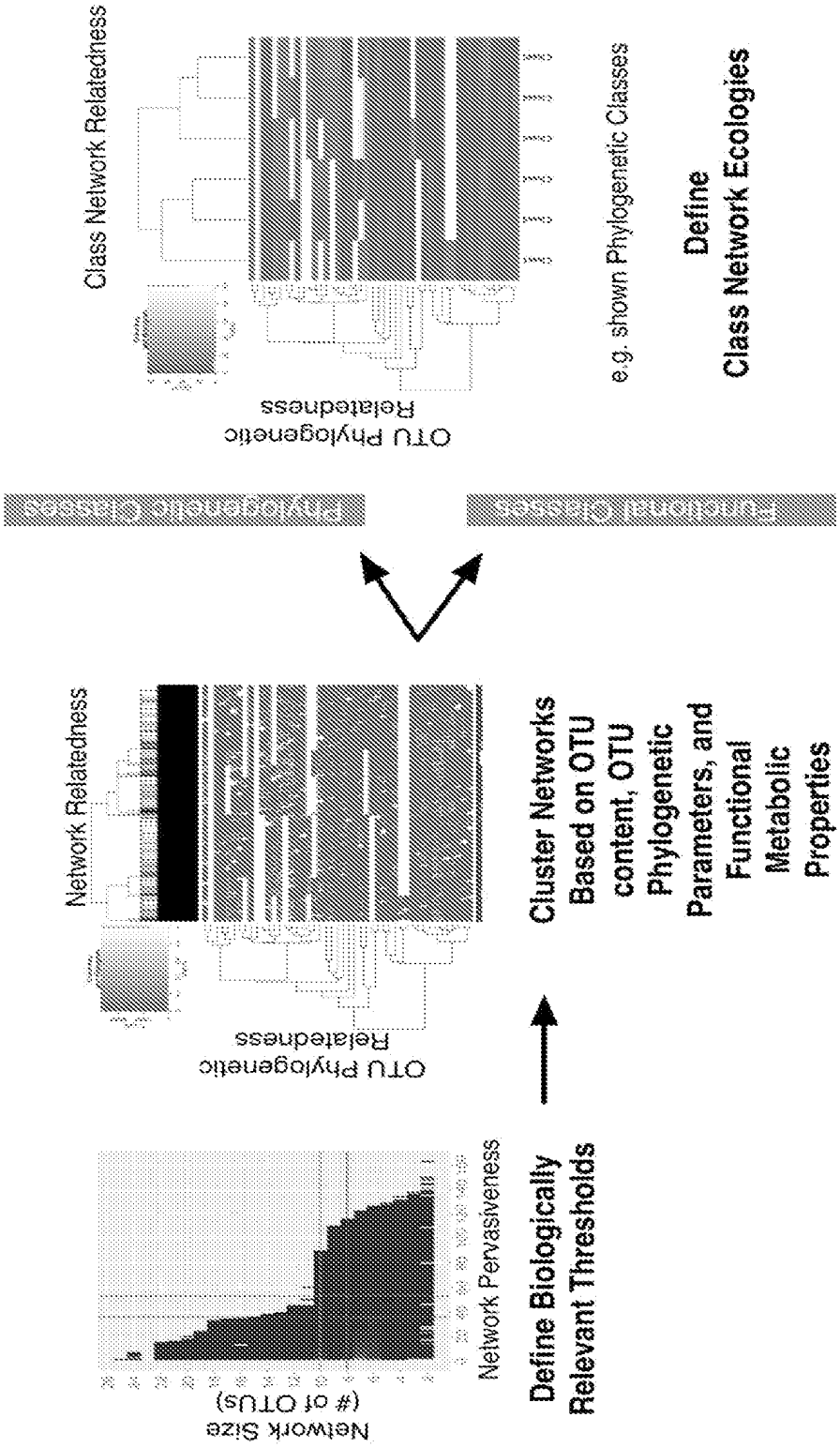
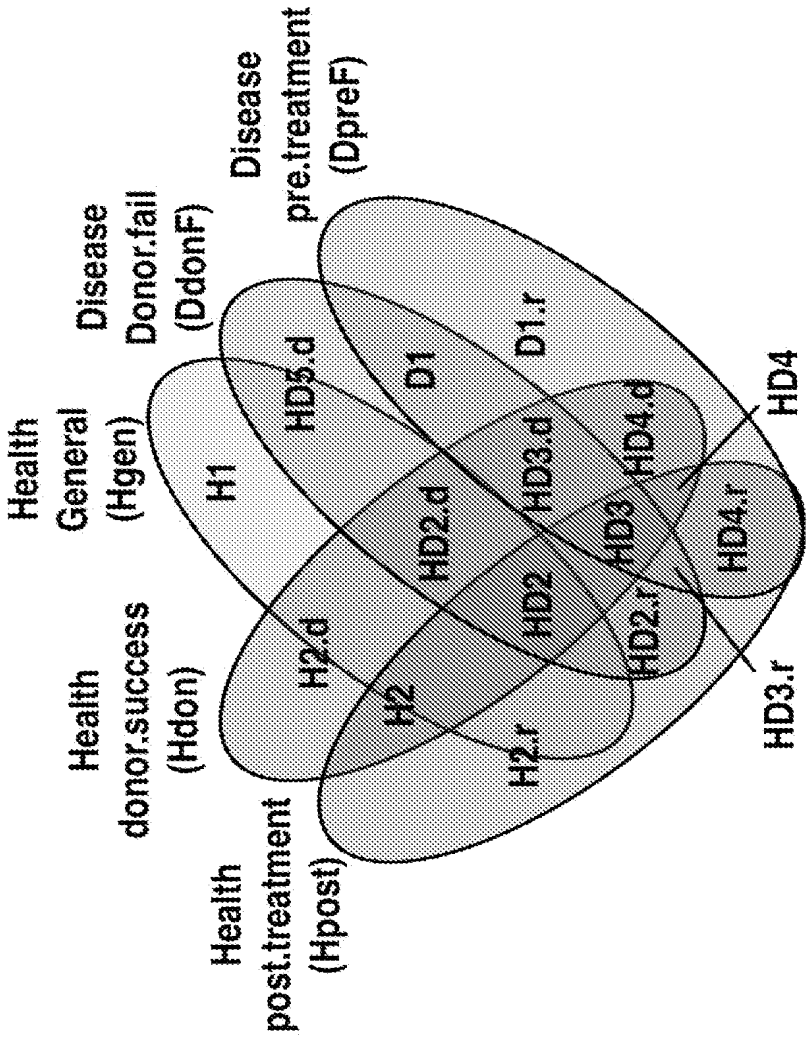


Figure 18


Health
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(DdonF)
Disease
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Health
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Figure 19

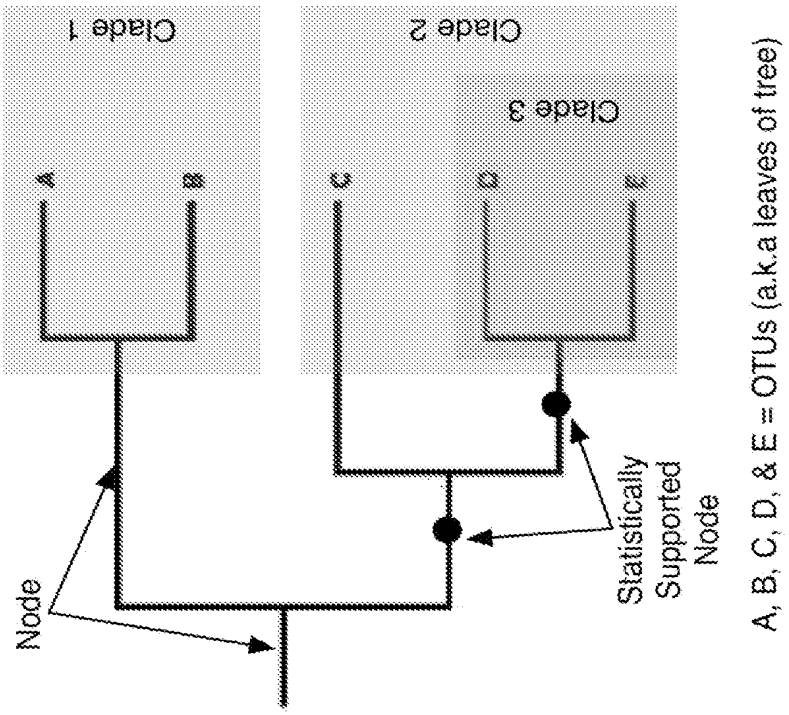

Figure 20

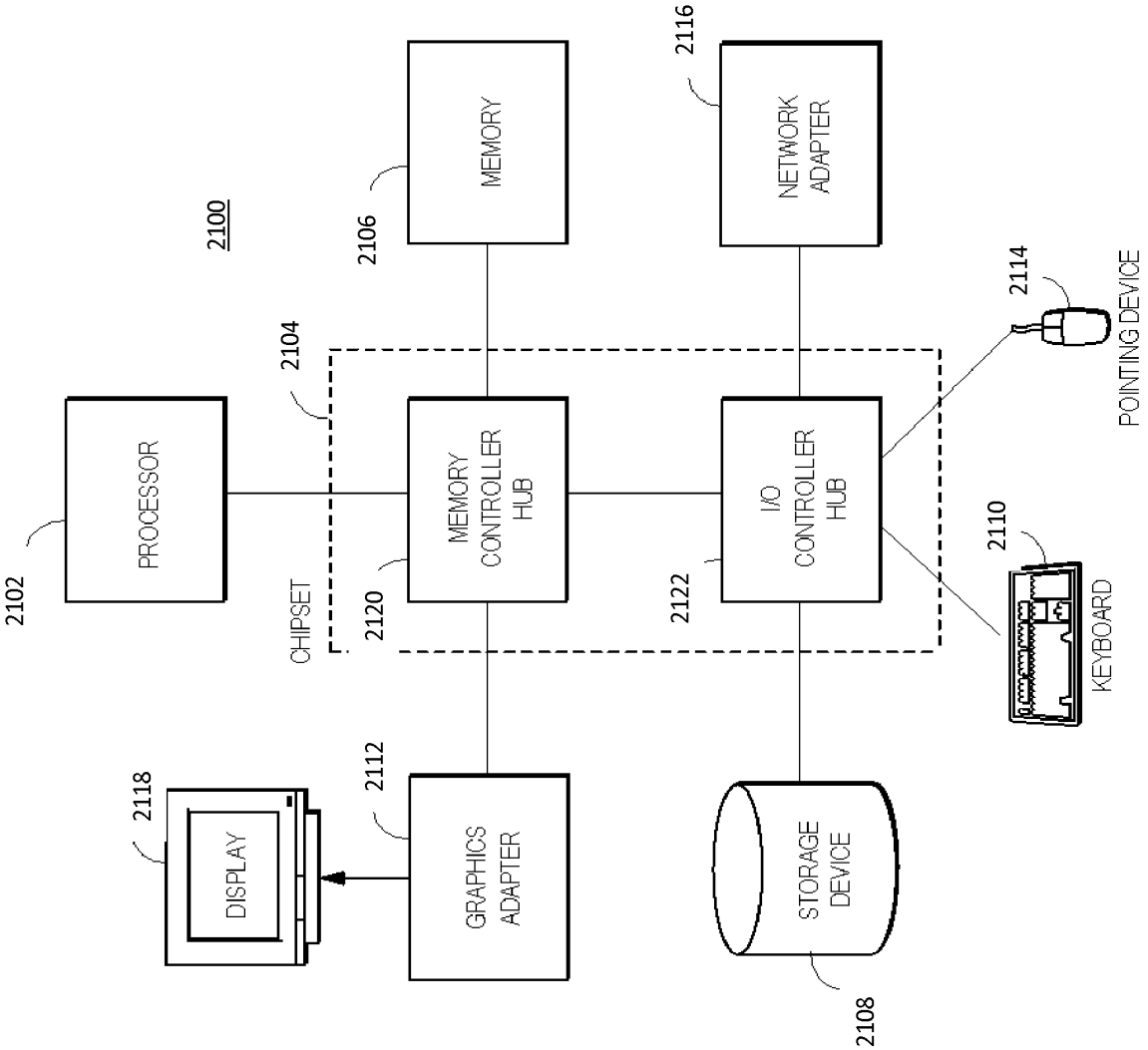

Figure 21

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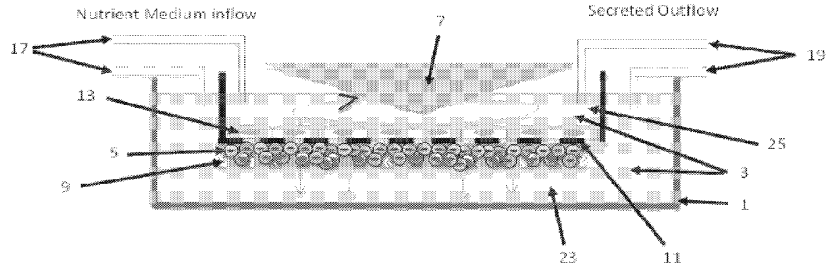
(54) Title: *IN VITRO* MODEL FOR A TUMOR MICROENVIRONMENT

FIG. 5



(57) Abstract: Methods for mimicking a tumor microenvironment *in vitro* are provided. The methods comprise indirectly applying a shear stress upon at least one tumor cell type plated on a surface within a cell culture container. Methods for mimicking tumor metastasis and methods for testing drugs or compounds in such systems are also provided.

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IN VITRO MODEL FOR A TUMOR MICROENVIRONMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/893,402, filed October 21, 2013, the entirety of which is herein incorporated by reference.

GOVERNMENT LICENSE RIGHTS

[0002] This invention was made with Government support under Contract Number HSN261201300024C awarded by the National Cancer Institute at the National Institutes of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention generally relates to methods for mimicking a tumor microenvironment *in vitro*. The present invention also relates to methods for testing drugs or compounds in such systems and identifying potential cancer drug targets.

BACKGROUND OF THE INVENTION

[0004] *In vivo*, the tumor microenvironment is a complex milieu containing multiple cell types including tumor cells, vascular cells such as endothelial cells, and stromal cells, such as fibroblasts. In addition, *in vivo*, these cells are exposed to blood flow and various biological transport conditions. *In vivo*, microvascular cells in a tumor are affected by blood flow and communicate with tumor and non-tumor cells, both physically and through diffusible factors. In addition, the tumor vasculature is abnormal, characterized by chaotic branching, a low flow rate, and leaky vessels, and thus serves as a major transport barrier to anticancer therapies that target tumor cells. The interplay between tumor cells, endothelial cells, and stromal cells affects each cell type, leading to increased angiogenesis and tumor cell proliferation, and this crosstalk may be an important factor in determining the responsiveness of tumor cells to anticancer drugs.

[0005] Conventional *in vitro* tumor models using static monocultures of tumor cells fail to adequately model *in vivo* tumor biology. Current *in vitro* tumor models also do not accurately predict efficacy and safety of anticancer therapies *in vivo*. Traditional *in vitro* studies performed under static conditions are generally poor predictors of drug sensitivity,

due to the lack of representation of components of the tumor microenvironment. Furthermore, the conventional models often do not exhibit responses to drugs or compounds at concentrations that produce the response *in vivo*, instead requiring much higher concentrations of the drug or compound to induce the same response. Thus, there exists a need in the art for methods for accurately mimicking the *in vivo* tumor microenvironment *in vitro*. Such methods would improve the accuracy of preclinical screening of anticancer agents for efficacy and safety.

SUMMARY OF THE INVENTION

[0006] A method for mimicking a tumor microenvironment *in vitro* is provided. The method comprises adding a culture medium to a cell culture container and plating at least one tumor cell type on a surface within the cell culture container. A shear stress is indirectly applied upon the at least one tumor cell type, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment. The flow is time-variant.

[0007] Another method for mimicking a tumor microenvironment *in vitro* is also provided. The method comprising adding a culture medium to a cell culture container and plating at least one tumor cell type on a first surface of a porous membrane within the cell culture container. A shear stress is indirectly applied upon the at least one tumor cell type by applying a shear stress upon a second surface of the porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

[0008] Yet another method for mimicking a tumor microenvironment *in vitro* is also provided. The method comprises adding a culture medium to a cell culture container and plating at least one tumor cell type or stromal cell type on a first surface of a porous membrane within the cell culture container. When the stromal cell type is plated on the first surface of the porous membrane, at least one tumor cell type is present on a surface within the cell culture container. A shear stress is indirectly applied upon the at least one tumor cell type by applying a shear stress upon a second surface of the porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

[0009] A further method for mimicking a tumor microenvironment *in vitro* is also provided. The method comprises adding a culture medium to a cell culture container and

plating at least one stromal cell type on a first surface of a first porous membrane within the cell culture container. A second porous membrane is placed on the plated stromal cell type, such that a first surface of the second porous membrane contacts the plated stromal cells. At least one tumor cell type is plated on a second surface of the second porous membrane. A shear stress is indirectly applied upon the at least one tumor cell type by applying a shear stress upon the second surface of the first porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

[0010] An *in vitro* method of testing a drug or a compound for an effect on a tumor is provided. The method comprises mimicking the tumor microenvironment and adding a drug or a compound to the culture medium. A shear stress is indirectly applied upon the at least one tumor cell type directly or indirectly exposed to the drug or the compound. A change in the at least one tumor cell type, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on the tumor.

[0011] A method for mimicking tumor metastasis *in vitro* is also provided. The method comprises introducing cells of the at least one tumor cell type cultured according to any of the methods described above into an *in vitro* system that models an organ or tissue.

[0012] Another method for mimicking tumor metastasis is also provided. The method comprises introducing cells of the at least one tumor cell type cultured according to any of the methods described above into an animal.

[0013] Yet another method for mimicking tumor metastasis *in vitro* is provided. The method comprises adding a culture medium to a cell culture container and plating at least one cell type on a first surface of a porous membrane within the cell culture container, wherein the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one cell type and an upper volume comprising a second surface of the porous membrane. A shear stress is indirectly applied upon the at least one cell type, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the cells are indirectly exposed *in vivo*. Tumor cells derived from a human or a humanized animal are introduced into the upper volume or the lower volume.

[0014] An *in vitro* method of testing a drug or a compound for an effect on tumor metastasis is provided. The method comprises mimicking tumor metastasis *in vitro* and

adding a drug or a compound to the culture medium. A change in the cells of the at least one tumor cell type in the *in vitro* system that models the organ or tissue, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on tumor metastasis.

[0015] A method for selecting a chemotherapy regimen to be administered to a subject having a tumor is provided. The method comprises testing a drug or a compound *in vitro* for an effect on the tumor or testing a drug or a compound for an *in vitro* effect on tumor metastasis, wherein the at least one tumor cell type comprises tumor cells derived from the subject's tumor. The method further comprises determining whether to administer the drug or the compound to the subject based on the results of the *in vitro* testing.

[0016] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIG. 1 depicts a cone-and-plate device and indirect application of a shear stress to tumor cells.

[0018] FIG. 2 is a perspective of the clip that mounts on the cell culture container and secures inflow and outflow tubing to perfuse the upper and lower volumes.

[0019] FIG. 3 shows the positioning of two clips in a cell culture container.

[0020] FIG. 4A shows a Doppler sonography image of the central bronchial artery in a patient diagnosed with a pulmonary lesion.

[0021] FIG. 4B depicts wall shear stress calculations (dynes/cm^2) of the Doppler flow signal of a human pulmonary lesion.

[0022] FIG. 4C is a schematic illustration of an exemplary arterial blood supply in a pulmonary lesion. "PA" stands for pulmonary artery, "cBA" stands for central bronchial artery, and "pBA" stands for peripheral bronchial artery.

[0023] FIGs. 5–10 are schematic diagrams illustrating methods for mimicking a tumor microenvironment *in vitro*.

[0024] FIG. 11 shows an exemplary system that models the liver, which can be used to mimic tumor metastasis *in vitro*.

[0025] FIGs. 12A–D depict exemplary configurations for modeling tumor metastasis *in vitro* by pumping culture medium out of a cell culture container comprising at least one tumor cell type and into an *in vitro* system that models the liver.

[0026] FIGs. 13A–C provide fluorescent microscopy images for human dermal microvascular endothelial cells (FIG. 13A), human fibroblasts (FIG. 13B), and A549 human non-small cell carcinoma (NSCLC) tumor cells (FIG. 13C) cultured using the method depicted in FIG. 9 under hemodynamic shear stress. FIGs. 13D–F provide fluorescent microscopy images for A549 tumor cells cultured using the method depicted in FIG. 9 under hemodynamic shear stress in the substantial absence of exogenously added extracellular matrix (ECM) (FIG. 13D), where the A549 tumor cells were plated on a layer of collagen (FIG. 13E), or where the A549 tumor cells were plated on a layer of collagen and another layer of collagen was deposited on top of the plated A549 tumor cells such that the collagen substantially surrounded the tumor cells (FIG. 13F, "collagen sandwich").

[0027] FIG. 14A provides results from an assay measuring cell growth of A549 tumor cells under two-dimensional static conditions or using the method depicted in FIG. 9.

[0028] FIG. 14B provides a schematic illustration of the qualitative differences in the growth rate of tumor cells cultured in static two-dimensional cultures ("*In Vitro*"), in the *in vitro* tumor microenvironments described herein ("*In vitro* tumor microenvironment"), and in xenografts.

[0029] FIG. 14C provides results from an assay measuring the growth of A549 tumor cells cultured using the method depicted in FIG. 9 under hemodynamic shear stress in the substantial absence of exogenously added extracellular matrix ("no matrix"), where the A549 tumor cells were plated on a single layer of collagen ("collagen layer"), or where the A549 tumor cells were plated on a layer of collagen and another layer of collagen was deposited on top of the plated A549 tumor cells such that the collagen substantially surrounded the tumor cells ("collagen sandwich").

[0030] FIG. 15 provides results from a permeability assay assessing the permeability of endothelial cells cultured in the presence ("tumor cells") or absence ("no tumor cells") plated on the opposing side of a porous membrane and cultured under hemodynamic shear stress.

[0031] FIGs. 16A and 16B provide a dendogram (FIG. 16A) and a heatmap (FIG. 16B) showing the expression and clustering of 14,159 genes in A549 tumor cells grown under static two-dimensional conditions ("plastic"), in xenografts, or using the method shown in FIG. 9 under hemodynamic shear stress in the substantial absence of exogenously added extracellular matrix ("NC" or "no collagen"), where the A549 tumor cells were plated on a single layer of collagen ("CL" or "collagen layer") or where the A549 tumor cells were

plated on a layer of collagen and another layer of collagen was deposited on top of the plated A549 tumor cells such that the collagen substantially surrounded the tumor cells ("CS" or "collagen sandwich").

[0032] FIGs. 17A and 17B provide a dendogram (FIG. 17A) and a heatmap showing the expression and clustering of 7935 genes differentially expressed between xenografts and static two-dimensional cultures of A549 tumor cells in A549 tumor cells grown in xenografts or using the method depicted in FIG. 9 under hemodynamic shear stress in the substantial absence of exogenously added extracellular matrix (NC) or in a collagen sandwich (CS), as compared to static-two dimensional cultures ("plastic").

[0033] FIGs. 18A and 18B provide a dendogram (FIG. 18A) and a heatmap (Fig. 18B) showing the clustering of 48 genes annotated with "non-small cell lung cancer" in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database in A549 tumor cells grown in xenografts or using the method depicted in FIG. 9 under hemodynamic shear stress in the substantial absence of exogenously added extracellular matrix (NC) or in a collagen sandwich (CS), as compared to static-two dimensional cultures ("plastic").

[0034] FIGs. 19A and 19B provide results showing the inhibition of the growth of A549 tumor cells in the presence of cisplatin, MK2206, or selumetinib (AZD6244).

[0035] FIGs. 20A–F are fluorescent microscopy images of hepatocytes cultured under static conditions or in the presence of controlled hemodynamics.

[0036] FIG. 21A is a fluorescent microscopy image of hepatocytes cultured under controlled hemodynamics.

[0037] FIG. 21B is a fluorescent microscopy image of *in vivo* liver.

[0038] FIG. 21C shows transmission electron microscopy images of hepatocytes cultured under controlled hemodynamics.

[0039] FIGs. 22A–B provide data for albumin and urea secretion in hepatocytes cultured under static conditions or controlled hemodynamics.

[0040] FIGs. 23A–D provide metabolic gene expression data for hepatocytes cultured under static conditions or controlled hemodynamics.

[0041] FIGs. 24A–B provide cytochrome p450 activity data for hepatocytes cultured under static conditions or controlled hemodynamics.

[0042] FIG. 24C is a fluorescent microscopy image from an assay for transporter activity in hepatocytes cultured under controlled hemodynamics.

[0043] FIG. 25 shows gene expression data for an *in vitro* fatty liver model.

[0044] FIG. 26 shows gene expression data for an *in vitro* fatty liver model.

[0045] FIGs. 27A–B provide fluorescent microscopy images of hepatocytes cultured under healthy conditions or conditions that mimic fatty liver disease.

[0046] FIG. 28 shows a transmission electron microscopy image of rat hepatocytes cultured under high glucose/high insulin conditions.

[0047] FIGs. 29A–B provide results from assays measuring total lipids and total triglycerides in hepatocytes cultured under healthy conditions or conditions that mimic fatty liver disease.

[0048] FIGs. 30A–B provide gene expression data for hepatocytes cultured under healthy conditions or conditions that mimic fatty liver disease.

[0049] FIGs. 31A–B provide metabolic gene expression data and cytochrome p450 activity data for hepatocytes cultured under healthy conditions or conditions that mimic fatty liver disease.

[0050] FIGs. 32A–3C show fluorescent microscopy images from hepatocytes cultured under healthy conditions or under conditions that mimic fatty liver disease, in the presence or absence of pioglitazone.

[0051] FIG. 33 provides results from an assay measuring total triglycerides in hepatocytes cultured under healthy conditions or under conditions that mimic fatty liver disease, in the presence or absence of pioglitazone.

[0052] FIG. 34 provides metabolic gene expression data for hepatocytes cultured under healthy conditions or under conditions that mimic fatty liver disease, in the presence or absence of pioglitazone.

[0053] FIGs. 35A–C provide cytochrome activity data for hepatocytes cultured under controlled hemodynamic conditions or static conditions in the presence of phenobarbital or rifampicin.

[0054] FIG. 36A provides fluorescence microscopy images showing the toxicity response of hepatocytes cultured under controlled hemodynamic conditions to chlorpromazine at an *in vivo* plasma C_{max} concentration.

[0055] FIG. 36B provides data showing a toxicity dose-response for hepatocytes cultured under controlled hemodynamics or static conditions and exposed to varying concentrations of chlorpromazine.

[0056] FIGs. 37A–B provides data showing upregulation of oxidative stress-related toxicity genes (FIG. 37A) and metabolic genes (FIG. 37B) in response to chlorpromazine in hepatocytes cultured under controlled hemodynamic conditions.

[0057] FIG. 38 provides acute toxicity data, measured by release of miRNA122, in hepatocytes cultured under controlled hemodynamic or static conditions in response to chlorpromazine.

[0058] FIG. 39 provides fluorescence microscopy images showing sublethal toxicity and cholestatic changes in hepatocytes cultured under controlled hemodynamic conditions in response to treatment with troglitazone.

[0059] FIG. 40 provides data showing the upregulation of oxidative stress-related genes and MRP3 and MRP4 genes in hepatocytes cultured under controlled hemodynamic conditions in response to treatment with troglitazone.

[0060] FIG. 41A provides a fluorescence microscopy image showing retention of polarized morphology in canine hepatocytes cultured under controlled hemodynamic conditions.

[0061] FIG. 41B provides gene expression data showing expression of CYP1A1 and CYP3A1 in canine hepatocytes cultured under controlled hemodynamic conditions or static conditions.

[0062] FIG. 42 provides a fluorescence microscopy image showing retention of polarized morphology in hepatocytes derived from inducible pluripotent stem cells (iPSCs) cultured under controlled hemodynamic conditions.

FIGs. 43A–C provide gene expression data showing the expression of metabolic genes and differentiation genes in iPSC-derived hepatocytes cultured under controlled hemodynamic conditions.

[0063] Corresponding reference characters indicate corresponding parts throughout the drawings.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0064] The present invention provides methods for mimicking a tumor microenvironment *in vitro*. In contrast to the static monoculture models currently used as the standard *in vitro* models of tumor biology by the pharmaceutical and biopharmaceutical industries, the methods of the present invention recreate the tumor microenvironment and can be used to assess multiple aspects of cancer, including endothelial cell barrier function, tumor

growth, cell proliferation, cell migration, cell invasion, and alterations in responsiveness of tumor cells to anticancer therapies.

[0065] A method for mimicking a tumor microenvironment *in vitro* is provided. The method comprises adding a culture medium to a cell culture container, plating at least one tumor cell type on a surface within the cell culture container, and indirectly applying a shear stress upon the at least one tumor cell type. The shear stress results from flow of the culture medium induced by a flow device. The flow mimics flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment. The flow is time-variant.

[0066] At least one extracellular matrix component can be deposited on the surface within the cell culture container, and the at least one tumor cell type can be plated on the at least one extracellular matrix component. Alternatively, the at least one tumor cell type can be suspended in a solution comprising at least one extracellular matrix component to create a suspension comprising the at least one tumor cell type and the at least one extracellular matrix component. The suspension can then be deposited on the surface within the cell culture container. The shear stress can be indirectly applied upon the at least one extracellular matrix component and the at least one tumor cell type.

[0067] The method can further comprise plating the at least one tumor cell type on a first surface of a porous membrane and indirectly applying the shear stress upon the at least one tumor cell type by applying the shear stress upon a second surface of the porous membrane.

[0068] Another method for mimicking a tumor microenvironment *in vitro* is also provided. The method comprises adding a culture medium to a cell culture container, plating at least one tumor cell type on a first surface of a porous membrane within the cell culture container, and indirectly applying a shear stress upon the at least one tumor cell type by applying a shear stress upon a second surface of the porous membrane. The shear stress results from flow of the culture medium induced by a flow device. The flow mimics flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

[0069] Yet another method for mimicking a tumor microenvironment *in vitro* is provided. The method comprises adding a culture medium to a cell culture container and plating at least one tumor cell type or stromal cell type on a first surface of a porous membrane within the cell culture container. When the stromal cell type is plated on the first surface of the porous membrane, at least one tumor cell type is present on a surface within the cell culture container. Shear stress is indirectly applied upon the at least one tumor cell type

by applying a shear stress upon a second surface of the porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

[0070] In any of the methods wherein the at least one tumor cell type is plated on a first surface of a porous membrane, the porous membrane can be suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one tumor cell type and an upper volume comprising a second surface of the porous membrane. The shear stress is applied upon the second surface of the porous membrane in the upper volume of the container.

[0071] In addition, in any of the methods wherein the at least one tumor cell type is plated on a first surface of a porous membrane, the method can further comprise depositing at least one extracellular matrix component on the first surface of the porous membrane and plating the at least one tumor cell type on the at least one extracellular matrix component. Alternatively, the at least one tumor cell type can be suspended in a solution comprising at least one extracellular matrix component to create a suspension comprising the at least one tumor cell type and the at least one extracellular matrix component, and the suspension can be deposited on the first surface of the porous membrane. The porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one extracellular matrix component and the at least one tumor cell type, and an upper volume comprising a second surface of the porous membrane. The shear stress is applied upon the second surface of the porous membrane in the upper volume of the container.

[0072] The method can further comprise plating endothelial cells on the second surface of the porous membrane, and applying the shear stress upon the plated endothelial cells. The at least one tumor cell type is plated on a first surface of the porous membrane.

[0073] The method can further comprise plating at least one stromal cell type on the second surface of the porous membrane and applying the shear stress upon the plated stromal cell type.

[0074] In methods where the at least one stromal cell type is plated on the second surface of the porous membrane, the method can further comprise plating endothelial cells on the second surface of the porous membrane. The at least one stromal cell type can be mixed

with the endothelial cells prior to plating, and the method can comprise applying the shear stress upon the plated mixture of the at least one stromal cell type and the endothelial cells. Alternatively, the at least one stromal cell type and the endothelial cells can be sequentially plated on the second surface of the porous membrane. For example, the method can comprise plating the at least one stromal cell type on the second surface of the porous membrane, subsequently plating the endothelial cells on the plated stromal cell type, and applying the shear stress on the plated endothelial cells. Alternatively, the method can comprise plating the endothelial cells on the second surface of the porous membrane, subsequently plating the at least one stromal cell type on the plated endothelial cells, and applying the shear stress on the plated stromal cell type.

[0075] In the methods wherein the at least one tumor cell type is plated on a first surface of a porous membrane, the porous membrane can be a first porous membrane and the method can comprise plating the at least one tumor cell type on a first surface of the first porous membrane. At least one stromal cell type is plated on a second surface of the first porous membrane. A second porous membrane is placed on the plated stromal cell type such that a first surface of the second porous membrane contacts the plated stromal cells. The shear force is applied upon a second surface of the second porous membrane.

[0076] In the methods comprising depositing at least one extracellular matrix component or a suspension comprising the at least one tumor cell type and at least one extracellular matrix component on the first surface of a porous membrane the porous membrane can be a first porous membrane and the method can further comprise depositing the at least one extracellular matrix component on the first surface of the first porous membrane and plating the at least one tumor cell type on the at least one extracellular matrix component. Alternatively, the method can further comprise depositing the suspension comprising the at least one tumor cell type and the at least one extracellular matrix component on the first surface of the first porous membrane. Either method further comprises plating at least one stromal cell type on a second surface of the first porous membrane, placing a second porous membrane on the plated stromal cell type such that a first surface of the second porous membrane contacts the plated stromal cells, and applying the shear force upon a second surface of the second porous membrane.

[0077] In the methods wherein the porous membrane is a first porous membrane described above, the method can further comprise plating endothelial cells on the second

surface of the second porous membrane and applying the shear force upon the plated endothelial cells.

[0078] In the methods that comprise plating at least one tumor cell type or stromal cell type on a first surface of a porous membrane, the porous membrane can be a first porous membrane and the method can further comprise plating the at least one stromal cell type on a first surface of a first porous membrane. A second porous membrane is placed on the plated stromal cell type, such that a first surface of the second porous membrane contacts the plated stromal cells. At least one tumor cell type is plated on a second surface of the second porous membrane. The shear force is indirectly applied upon the at least one tumor cell type by applying the shear stress upon the second surface of the first porous membrane.

[0079] The present invention is also directed to another method for mimicking a tumor microenvironment *in vitro*. The method comprises adding a culture medium to a cell culture container and plating at least one stromal cell type on a first surface of a first porous membrane within the cell culture container. A second porous membrane is placed on the plated stromal cell type, such that a first surface of the second porous membrane contacts the plated stromal cells. At least one tumor cell type is plated on a second surface of the second porous membrane. Shear stress is indirectly applied upon the at least one tumor cell type by applying a shear stress upon the second surface of the first porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

[0080] In the methods comprising plating at least one stromal cell type on a first surface of a first porous membrane, the first porous membrane can be suspended in the cell culture container such that the first surface of the first porous membrane is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one tumor cell type, the second porous membrane, and the at least one stromal cell type, and an upper volume comprising a second surface of the first porous membrane. The shear stress is applied upon the second surface of the first porous membrane in the upper volume of the container.

[0081] In the methods comprising plating at least one stromal cell type on a first surface of a first porous membrane, the method can further comprise depositing at least one extracellular matrix component on the second surface of the second porous membrane and plating the at least one tumor cell type on the at least one extracellular matrix component. Alternatively, the method can further comprise suspending the at least one tumor cell type in

a solution comprising at least one extracellular matrix component to create a suspension comprising the at least one tumor cell type and the at least one extracellular matrix component, and depositing the suspension on the second surface of the second porous membrane.

[0082] In the methods comprising plating at least one stromal cell type on a first surface of a first porous membrane, the method can further comprise plating endothelial cells on the second surface of the first porous membrane and applying the shear stress upon the plated endothelial cells.

[0083] In the methods comprising the use of a second porous membrane, the method can further comprise immersing the second porous membrane in a solution comprising at least one extracellular matrix component prior to placing the second porous membrane on the plated stromal cell type.

[0084] In any of the methods comprising plating endothelial cells, the method can further comprises coplating at least one tumor cell type with the endothelial cells. The coplating can comprise mixing the at least one tumor cell type with the endothelial cells prior to plating. Alternatively, the coplating can comprise sequentially plating the at least one tumor cell type and the endothelial cells. For example, the coplating can comprise plating the at least one tumor cell type and subsequently plating the endothelial cells. Alternatively, the coplating can comprise plating the endothelial cells and subsequently plating the at least one tumor cell type.

[0085] In any of the methods comprising coplating at least one tumor cell type with the endothelial cells, the coplating can comprise plating the endothelial cells and the at least one tumor cell type at a ratio of about 100:1 to about 3:1. For example, the coplating can comprise plating the endothelial cells and the at least one tumor cell type at a ratio of about 50:1 to about 10:1.

[0086] In any of the methods comprising coplating at least one tumor cell type with the endothelial cells, the at least one tumor cell type can be any of the tumor cell types described herein. In these methods, the at least one tumor cell type preferably comprises cells derived from a glioblastoma.

[0087] In any of the methods wherein cells are plated on a porous membrane, the porous membrane, the first porous membrane, or the second porous membrane can adapted to permit fluid communication of the culture medium and physical interaction and communication between cells plated on opposing sides of the porous membrane.

[0088] The present invention also relates to an *in vitro* method of testing a drug or a compound for an effect on a tumor. The method comprises mimicking the tumor microenvironment, adding a drug or a compound to the culture medium, and indirectly applying the shear stress upon the at least one tumor cell type directly or indirectly exposed to the drug or the compound. A change in the at least one tumor cell type, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on the tumor. The tumor microenvironment can be mimicked by the *in vitro* methods of mimicking a tumor microenvironment described above.

[0089] To confirm that the *in vivo* tumor microenvironment is mimicked, a change in the level or localization of a marker of the tumor microenvironment can be compared between a method of the invention and the same method in the absence of the application of the shear stress. The level or localization of the marker in the at least one tumor cell type, the at least one stromal cell type, or the endothelial cells upon application of the shear stress is compared to the level or localization of the marker in the at least one tumor cell type, the at least one stromal cell type, or the endothelial cells in the absence of the application of shear stress. Alternatively, the level of a marker in the culture medium upon application of the shear stress is compared to the level of the marker in the culture medium in the absence of the application of the shear stress. For example, if a marker is known to be associated with the presence of a tumor and its concentration is known to increase in the serum when a tumor is present *in vivo*, an increase in the level of the marker in the culture medium of the method of the invention with application of the shear stress as compared to the level of the marker in the culture medium in the absence of the application of the shear stress confirms that the tumor microenvironment is mimicked by the *in vitro* method of the invention.

[0090] In any of the above methods, the cell culture container can comprise inlets and outlets. The inlets and outlets can be used for perfusing cell culture medium, drugs, compounds, and other components into and out of the cell culture container.

[0091] The inlets and outlets can be within the portions of the cell culture container defining the upper and lower volumes.

[0092] Any of the methods can comprise perfusing culture medium into and out of the cell culture container. For example, the methods can comprise perfusing culture medium into and out of the upper volume and/or perfusing culture medium into and out of the lower volume.

[0093] Any of the methods for mimicking a tumor microenvironment *in vitro* can comprise mimicking the tumor microenvironment *in vitro* in a first cell culture container according to any of the methods described herein, mimicking the tumor microenvironment *in vitro* in a second cell culture container according to any of the methods described herein, and transferring cells of the at least one tumor cell type cultured in the first cell culture container into the second cell culture container. The transferring can comprise manually transferring the cells of the at least one tumor cell type cultured in the first cell culture container into the second cell culture container. Alternatively, an outlet in the first cell culture container can be connected to an inlet in the second cell culture container, and the method can further comprise pumping culture medium comprising the at least one tumor cell type out of the first cell culture container and into the second cell culture container.

[0094] In any of the methods for mimicking a tumor microenvironment *in vitro* described herein, the method can further comprise introducing cells cultured in an *in vitro* system that models an organ or tissue into the cell culture container.

Cell Types

[0095] Any of the methods described above can further comprise plating at least one stromal cell type on the surface within the cell culture container, on the at least one extracellular matrix component, or on the first surface of the porous membrane. Alternatively, at least one stromal cell type can be suspended with the tumor cell type in the solution comprising the at least one extracellular matrix component to create a suspension comprising the at least one stromal cell type, the at least one tumor cell type, and the at least one extracellular matrix component, and the suspension can be deposited on the surface within the cell culture container or on the first surface of the porous membrane.

[0096] For methods wherein the at least one tumor cell type is plated on a first surface of a first porous membrane, the methods can further comprise plating at least one stromal cell type on the first surface of the first porous membrane. Alternatively, the methods can further comprise suspending at least one stromal cell type with the tumor cell type in the solution comprising the at least one extracellular matrix component to create a suspension comprising the at least one stromal cell type, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the first surface of the first porous membrane.

[0097] For methods wherein the at least one tumor cell type is plated on a second surface of a second porous membrane, the methods can further comprise plating at least one stromal cell type on the second surface of the second porous membrane. Alternatively, the methods can further comprise suspending at least one stromal cell type with the tumor cell type in the solution comprising the at least one extracellular matrix component to create a suspension comprising the at least one stromal cell type, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the second surface of the second porous membrane.

[0098] Any of the methods described above can also further comprise plating one or more additional cell types on a surface of the cell culture container, on the at least one extracellular matrix component, on the first or second surface of the porous membrane, on the first or second surface of the first porous membrane, or on the first or second surface of the second porous membrane; or suspending one or more additional cell types in the culture medium within the upper volume or in the culture medium within the lower volume.

[0099] In any of the methods that comprise suspending the at least one tumor cell type in a solution comprising at least one extracellular matrix component, the method can further comprise suspending one or more additional cell types with the at least one tumor cell type in the solution comprising the at least on extracellular matrix component to create a suspension comprising the one or more additional cell types, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the surface within the cell culture container, on the first surface of the porous membrane, on the first surface of the first porous membrane, or on the second surface of the second porous membrane.

[00100] Cell types for use in the methods of the invention include primary cells and immortalized cells. The at least one tumor cell type, the endothelial cells, the at least one stromal cell type, or the one or more additional cell types can comprise immortalized cells. The at least one tumor cell type, the endothelial cells, the at least one stromal cell type, or the one or more additional cell types can comprise primary cells.

[00101] Any of the cell types can comprise cells derived from an animal, e.g., from a genetically modified animal or a human.

[00102] In any of the methods of the invention, the method can further comprise the step of culturing the cell type or cell types.

[00103] The at least one tumor cell type, the endothelial cells, the at least one stromal cell type, and additional cell types that can be used in the methods are further described below.

Tumor Cells

[00104] The at least one tumor cell type can comprise cells derived from a carcinoma, a sarcoma, a lymphoma, an adenosquamous carcinoma, a mixed mesodermal tumor, carcinosarcoma, a teratocarcinoma, or a combination thereof.

[00105] The cells derived from a carcinoma can comprise cells derived from an adenocarcinoma, cells derived from a squamous cell carcinoma, or a combination thereof.

[00106] The cells derived from a sarcoma can comprise cells derived from an osteosarcoma, a chondrosarcoma, a leiomyosarcoma, a rhabdomyosarcoma, a mesothelial sarcoma (mesothelioma), a fibrosarcoma, an angiosarcoma (e.g., from a hemangioendothelioma, a lymphangiosarcoma, or a combination thereof), a liposarcoma, a glioma, an astrocytoma, a myxosarcoma, a mesenchymous tumor, a mixed mesodermal tumor, or a combination thereof.

[00107] The cells derived from a lymphoma can comprise cells derived from a Hodgkin lymphoma, a non-Hodgkin lymphoma, or a combination thereof.

[00108] The at least one tumor cell type can be derived from a tumor of connective tissue, a tumor of endothelium or mesothelium, a tumor of lymphoid tissue, a tumor of muscle, a tumor of an epithelial tissue, a tumor of a neural tissue, a tumor of the amine precursor uptake and decarboxylation (APUD) system, a tumor of a neural crest-derived cell, a gonadal tumor, or a combination thereof.

[00109] Where the at least one tumor cell type is derived from a tumor of connective tissue, the tumor can comprise a tumor of adult fibrous tissue (e.g., a firboma or a fibrosarcoma), embryonic (myxomatous) fibrous tissue (e.g., a myxoma or a myxosarcoma), adipose tissue (e.g., a lipoma or a liposarcoma), cartilage tissue (e.g., a chondroma or a chondrosarcoma), bone (e.g., osteoma or a osteosarcoma), notochord (e.g., a chordoma), a fibrous histiocytoma (e.g., a malignant fibrous histiocytoma), or a combination thereof.

[00110] Where the at least one tumor cell type is derived from a tumor of endothelium or mesothelium, the tumor can comprise a blood vessel tumor (e.g., a hemangioma, a hemangiopericytoma, a hemangiosarcoma, or an angiosarcoma), a lymph

vessel tumor (e.g., a lymphangioma or a lymphangiosarcoma), a mesothelium tumor (e.g., a mesothelioma), or a combination thereof.

[00111] Where the at least one tumor cell type is derived from a tumor of lymphoid tissue, the tumor can comprises a plasmacytoma, a Hodgkin lymphoma, a non-Hodgkin lymphoma, or a combination thereof.

[00112] Where the at least one tumor cell type is derived from a tumor of muscle, the tumor can comprise a smooth muscle tumor (e.g., a leiomyoma or a leiomyosarcoma), a striated muscle tumor (e.g., a rhabdomyoma or a rhabdomyosarcoma), or a combination thereof.

[00113] Where the at least one tumor cell type is derived from a tumor of an epithelial tissue, the tumor can comprise a tumor of a stratified squamous tissue (e.g., a papilloma, a seborrheic keratosis, a skin adnexal tumor, a squamous cell carcinoma, or an epidermoid carcinoma), a tumor of a glandular epithelium (e.g., a tumor of the glandular epithelium or a liver, kidney or bile duct), a tumor of transitional epithelium (e.g., a transitional cell papilloma or a transitional cell carcinoma), a placental tumor (e.g., a hydatidiform mole or a choriocarcinoma), a testicular tumor (e.g., a seminoma or an embryonal cell carcinoma), or a combination thereof. Where the tumor of the glandular epithelium is a tumor of the glandular epithelium of the liver, the tumor can comprise a hepatic adenoma or a hepatocellular carcinoma. Where the tumor of the glandular epithelium is a tumor of the glandular epithelium of the kidney, the tumor can comprise a renal tubular adenoma, a renal cell carcinoma, or a hypernephroma. Where the tumor of the glandular epithelium is a tumor of the glandular epithelium of the bile duct, the tumor can comprise a bile duct adenoma or a cholangiocarcinoma.

[00114] Where the at least one tumor cell type is derived from a tumor of a neural tissue, the tumor can comprise a glial cell tumor (e.g., a glioma or a glioblastoma), a nerve cell tumor (e.g., a ganglioneuroma, a nueroblastoma, or a medulloblastoma), a tumor of the meninges (e.g., a meningioma), a nerve sheath tumor (e.g., a Schwannoma, a neurilemmoma, a neurofibroma, a minigioma, or a neurofibrosarcoma), or a combination thereof.

[00115] Where the at least one tumor cell type is derived from a tumor of the amine precursor uptake and decarboxylation (APUD) system, the tumor can comprise a pituitary tumor (e.g., a basophilic adenoma, a eosinophilic adenoma, or a chromophobe adenoma), a parathyroid tumor (e.g., a parathyroid adenoma or a parathyroid carcinoma), a thyroid tumor (e.g., a C cell hyperplasia or a medullary carcinoma of the thyroid), a bronchial

lining tumor (e.g., a bronchial carcinoid or an oat cell carcinoma), an adrenal medulla tumor (e.g., a pheochromocytoma), a pancreatic tumor (e.g., an islet celladenoma, an insulinoma, a gastrinoma, or an islet cell carcinoma), a tumor of the stomach or intestines (e.g., a carcinoid), a tumor of the carotid body tumor or chemoreceptor system (e.g., a chemodectoma, a paraganglioma, or a carcinoid), or a combination thereof.

[00116] Where the at least one tumor cell type is derived from a tumor of a neural crest-derived cell, the tumor can comprise a tumor of a pigment producing cell (e.g., a nevus or a melanoma), a tumor of a Schwann cell of the peripheral nervous system (e.g., a Schwannoma or a neurilemmoma), a tumor of a Merkel cell (e.g., a Merkel cell neoplasm), or a combination thereof.

[00117] Where the at least one tumor cell type is derived from a gonadal tumor, the gonadal tumor can comprises a tumor of the ovary, a tumor of the testis, a seminoma, a dysgerminoma, a choriocarcinoma, an embryonal carcinoma, an endodermal sinus tumor, a teratocarcinoma, a Sertoli-Leydig cell tumor, an arrhenoblastoma, a granulosa-theca cell tumor, a hilar cell tumor, a lipid cell tumor, or a combination thereof.

[00118] The at least one tumor cell type can be derived from a tumor of the lung, breast, colon, rectum, prostate, bladder, bone, pancreas, liver, bile duct, ovary, testis, uterus, placenta, brain, cartilage, smooth muscle, striated muscle, membranous lining of a body cavity, fibrous tissue, blood vessel, lymph vessel, lymph node, adipose tissue, neurogenic connective tissue of the brain, kidney, pituitary gland, parathyroid, thyroid, bronchial lining, adrenalmedulla, stomach, large intestine, small intestine, carotid body, chemoreceptor system, skin, gall bladder, or a combination thereof.

[00119] The at least one tumor cell type can comprise immortalized cells. For example, the at least one tumor cell type can comprise an immortalized cell line comprising non-small cell lung adenocarcinoma cells, breast carcinoma cells, pancreas carcinoma cells, prostate cancer cells, ovarian carcinoma cells, colon cancer cells, or a combination thereof. For example, the immortalized cell line can comprise human non-small cell lung adenocarcinoma cell line A549, human breast carcinoma cell line MDA-MB-231, human pancreas carcinoma cell line BxPC-3, human prostate cancer cell line DU145, human prostate cancer cell line LNCaP, human ovarian carcinoma cell line SKOV-3, human colon cancer cell line COLO-205, or a combination thereof.

[00120] The at least one tumor cell type can comprise primary cells. For example, the tumor cell type can comprise primary tumor cells obtained from a subject by biopsy,

tumor resection, blood draw, or a combination thereof. A blood draw can be used to obtain cancer cells that have been shed from the primary tumor and that are present in the circulatory system. The primary tumor cells can be obtained from a stage I tumor, a stage II tumor, a stage III tumor, or a stage IV tumor.

[00121] The at least one tumor cell type can comprise tumor cells derived from a humanized animal bearing a tumor derived from a human subject, such as a humanized mouse. For example, the humanized mouse can be a non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse, a NOD/Shi-scid/IL-2Rγnull (NOG) mouse, or a NOD SCID IL-2Rγ knockout (NSG) mouse.

Endothelial Cells

[00122] The endothelial cells can comprise microvascular endothelial cells, macrovascular endothelial cells, endothelial progenitor cells, or a combination thereof.

[00123] The endothelial cells can be derived from a tumor. For example, where the at least one tumor cell type comprises cells derived from a tumor of an animal, the endothelial cells can be derived from the same tumor.

[00124] The endothelial cells can also be derived from an organ or tissue in which a tumor resides. For example, where the at least one tumor cell type comprises cells derived from a tumor of an animal, the endothelial cells can be derived from the organ or tissue in which that tumor resides. Thus, for instance, if the at least one tumor cell type comprises cells derived from a tumor of the lung, the endothelial cells can comprise endothelial cells derived from lung tissue of that animal or lung tissue of a different animal.

[00125] The endothelial cells can comprise endothelial cells derived from lung, breast, colon, rectum, prostate, bladder, bone, pancreas, liver, bile duct, ovary, testis, uterus, placenta, brain, cartilage, smooth muscle, striated muscle, a membranous lining of a body cavity, fibrous tissue, blood vessel, lymph vessel, lymph node, adipose tissue, neurogenic connective tissue of the brain, kidney, pituitary gland, parathyroid, thyroid, bronchial lining, adrenalmedulla, stomach, large intestine, small intestine, carotid body, chemoreceptor system, skin, gall bladder, or a combination thereof.

[00126] For example, the endothelial cells can comprise lung microvascular endothelial cells, breast microvascular endothelial cells, pancreatic microvascular endothelial cells, prostate microvascular endothelial cells, ovarian microvascular endothelial cells, colon microvascular endothelial cells, or a combination thereof.

[00127] The endothelial cells can comprise cells derived from inducible pluripotent stem cells (iPSC).

Stromal Cells

[00128] The at least one stromal cell type can comprise fibroblasts, immune cells, pericytes, inflammatory cells, or a combination thereof.

[00129] Where the at least one stromal cell type comprises fibroblasts, the fibroblasts can comprise fetal stromal fibroblasts, for example, human fetal stromal fibroblast cell line IMR-90. Alternatively, the fibroblasts can comprise human lung fibroblast cell line Hs888Lu.

[00130] Where the at least one stromal cell type comprises immune cells, the immune cells can comprise macrophages, lymphocytes, dendritic cells, or a combination thereof.

[00131] Where the at least one stromal cell type comprises inflammatory cells, the inflammatory cells can comprise B cells, T cells, or a combination thereof.

[00132] The at least one stromal cell type can comprise cells derived from inducible pluripotent stem cells (iPSC).

[00133] The at least one stromal cell type can be mixed with the at least one tumor cell type prior to plating. For example, the at least one stromal cell type can be mixed with the at least one tumor cell type at a ratio of about 0.1:1 to about 3:1, a ratio of about 0.2:1 to about 2:1, a ratio of about 0.25:1, or a ratio of about 1:1.

[00134] Alternatively, the method can comprise sequentially plating the at least one tumor cell type and the at least one stromal cell type. For example, the method can comprise plating the at least one tumor cell type and subsequently plating the at least one stromal cell type on the plated tumor cell type. Alternatively, the method can comprise plating the at least one stromal cell type and subsequently plating the at least one tumor cell type on the plated stromal cell type.

Additional Cell Types

[00135] The methods described herein can also further comprise plating one or more additional cell types on a surface of the cell culture container, on the at least one extracellular matrix component, on the first or second surface of the porous membrane, on the first or second surface of the first porous membrane, or on the first or second surface of hte

second porous membrane; or suspending one or more additional cell types in the culture medium within the upper volume or in the culture medium within the lower volume. For example, the one or more additional cell types can comprise a cell type adhered to the bottom surface of the cell culture container.

[00136] In any of the methods that comprise suspending the at least one tumor cell type in a solution comprising at least one extracellular matrix component, the method can further comprise suspending one or more additional cell types with the at least one tumor cell type in the solution comprising the at least on extracellular matrix component to create a suspension comprising the one or more additional cell types, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the surface within the cell culture container, on the first surface of the porous membrane, on the first surface of the first porous membrane, or on the second surface of the second porous membrane.

[00137] The one or more additional cell types can comprise fibroblasts, immune cells, pericytes, inflammatory cells, or a combination thereof.

[00138] Where the one or more additional cell types comprise fibroblasts, the fibroblasts can comprise fetal stromal fibroblasts, for example, human fetal stromal fibroblast cell line IMR-90. Alternatively, the fibroblasts can comprise human lung fibroblast cell line Hs888Lu.

[00139] Where the one or more additional cell types comprises immune cells the immune cells can comprise macrophages, lymphocytes, dendritic cells, or a combination thereof. For example, the immune cells can comprise the lymphocytes and the lymphocytes can be suspended in the culture medium within the upper volume.

[00140] Where the one or more additional cell types comprises inflammatory cells, the inflammatory cells can comprise B cells, T cells, or a combination thereof.

Extracellular Matrix Components

[00141] The one or more extracellular matrix components can be produced by a cell type plated on a surface within the cell culture container (e.g., by the at least one tumor cell type). When the extracellular matrix is produced by a cell type or cell types plated on a surface within the cell culture container, the extracellular matrix is referred to herein as "endogenous" extracellular matrix.

[00142] By contrast, when one or more extracellular matrix components are deposited on a surface within the cell culture container during the methods described herein, the extracellular matrix is referred to as "exogenous" or "exogenously added" extracellular matrix.

[00143] The methods described herein can comprise culturing the cell type or cell types in the substantial absence of exogenously added extracellular matrix. The "substantial absence of exogenously added extracellular matrix" means that the method does not comprise depositing an extracellular matrix component on a surface within the cell culture container, or suspending one or more cell types in a solution comprising an extracellular matrix component to create a suspension comprising the cell type and the at least one extracellular matrix component and depositing the suspension on a surface within the cell culture container. However, where the methods comprise the use of first and second porous membranes, the method can comprise immersing the second porous membrane in a solution comprising at least one extracellular matrix component prior to placing the second porous membrane on the plated stromal cell type. Without being bound to any particular theory, it is thought that when this immersion step is performed, the extracellular matrix component is absorbed by the porous membrane and aids in the attachment of cells to the membrane, but only results in the addition of a very small amount of extracellular matrix to the cell culture container. Thus, cells can be cultured in the "substantial absence of exogenously added extracellular matrix" even where this immersion step has been performed. Culturing cells in the "substantial absence of exogenously added extracellular matrix" also includes methods that do not comprise adding any exogenous extracellular matrix whatsoever to the cell culture container.

[00144] Extracellular matrix components for use in the methods of the invention can comprise a collagen, heparan sulfate, chondroitin sulfate, keratan sulfate, hyaluronic acid, an elastin, a fibronectin, a laminin, a vitronectin, or a combination thereof. The extracellular matrix component is preferably a type of extracellular matrix component that is present in the *in vivo* environment of the tumor cells, endothelial cells, stromal cells, and/or one or more additional cell types.

[00145] For example, where the extracellular matrix component comprises a collagen, the collagen can comprise collagen type I, collagen type II, collagen type III, collagen type IV, collagen type V, collagen type VI, collagen type VII, collagen type VIII, collagen type IX, collagen type X, collagen type XI, collagen type XII, collagen type XIII, collagen type XIV, collagen type XV, collagen type XVI, collagen type XVII, collagen type

XVIII, collagen type XIX, collagen type XX, collagen type XXI, collagen type XXII, collagen type XXIII, collagen type XXIV, collagen type XXV, collagen type XXVI, collagen type XXVII, collagen type XXVIII, or a combination thereof. Where the extracellular matrix component comprises a collagen, the concentration of the collagen is preferably about 1 mg/ml to about 10 mg/ml, about 2 mg/ml to about 5 mg/ml, or at least about 2 mg/ml.

[00146] The extracellular matrix component can comprise decellularized extracellular matrix purified from a biological source (e.g., human placenta).

[00147] The extracellular matrix component can be secreted by a cell type or cell types within the cell culture container (e.g., by the at least one tumor cell type).

[00148] The extracellular matrix component can be secreted by fibroblasts, chondrocytes, or osteoblasts plated on a surface within the cell culture container.

[00149] The extracellular matrix component suitably mimics the stiffness of the *in vivo* tumor microenvironment. For example, the at least one extracellular matrix component can have a Young's modulus of about 0.1 kPa to about 25 kPa, about 0.15 kPa to about 15 kPa, or about 3 kPa to about 12 kPa.

[00150] The at least one extracellular matrix component can have a non-uniform Young's modulus. For example, two or more different types of an extracellular matrix component or two or more concentrations of a single extracellular matrix component can be deposited on the surface within the cell culture container or the surface of the porous membrane to create a layer of extracellular matrix that has a non-uniform Young's modulus. The Young's modulus of the extracellular matrix can vary in a linear gradient across the surface within the cell culture container or across the surface of the porous membrane. Alternatively the Young's modulus of the extracellular matrix can vary in a concentric gradient on the surface within the cell culture container or the surface of the porous membrane.

[00151] The methods of the invention can also comprise depositing an additional layer of at least one extracellular matrix component on top of the at least one tumor cell type, such that the at least one extracellular matrix component substantially surrounds the at least one tumor cell type. The additional layer of at least one extracellular matrix component can have a Young's modulus that is different from the Young's modulus of the at least one extracellular matrix component deposited on the surface within the cell culture container, on the first surface of the porous membrane, on the first surface of the first porous membrane, or on the second surface of the second porous membrane. Alternatively, the additional layer of

at least one extracellular matrix component can have a Young's modulus that is substantially the same as the Young's modulus of the at least one extracellular matrix component deposited on the surface within the cell culture container, on the first surface of the porous membrane, on the first surface of the first porous membrane, or on the second surface of the second porous membrane. The Young's modulus of the additional layer of at least one extracellular matrix component can also be non-uniform.

[00152] In any of the methods that comprise the addition of one or more exogenous extracellular matrix components, the method can comprise the addition of single or multiple layers of ECM.

[00153] In any of the methods that comprise the addition of one or more exogenous extracellular matrix components, the exogenous extracellular matrix can comprise a single ECM protein or a mixture of multiple ECM proteins. For example, the exogenous extracellular matrix can comprise a mixture of collagen, fibronectin, and/or laminin.

Cell Culture Medium

[00154] Standard culture medium can be used in the methods of the invention. The composition of the culture medium will vary depending on the particular cell type(s) being cultured.

[00155] Additional components can also be included in the culture medium. For example, factors that are known to influence adipogenesis, such as GM-CSF and TGF-β, can be added to the culture medium. *In vivo*, these factors are secreted by macrophages.

[00156] The culture medium can comprise sera, blood, blood cells, a blood component, immune cells, conditioned culture medium, or a combination thereof.

[00157] The sera, blood, blood cells, blood component, or immune cells can be derived from a human or an animal (e.g., a mouse, rat, guinea pig, hamster, rabbit, cat, dog, monkey, cow, pig, horse, goat, sheep, bird, or fish).

[00158] The immune cells can comprise B cells, dendritic cells, granulocytes, innate lymphoid cells, megakaryocytes, monocytes, macrophages, natural killer cells, T cells, thymocytes, or a combination thereof.

[00159] The blood cells can comprise platelets, red blood cells, or a combination thereof.

[00160] The blood component can comprises a clotting factor, a lipoprotein, a triglyceride, or a combination thereof.

[00161] The conditioned culture medium can comprises conditioned culture medium from a culture comprising tumor cells, a culture comprising endothelial cells, a culture comprising a stromal cell type, or a combination thereof.

Flow Devices

[00162] The shear stress can be applied using any suitable flow device which is capable of inducing flow of the culture media, wherein the flow mimics flow to which the cell type or cell types being cultured are exposed *in vivo* in the tumor microenvironment. For example, the flow device can be a cone-and-plate device or a parallel plate flow device.

[00163] The flow device can be a cone-and-plate device substantially as described in U.S. Patent No. 7,811,782 and in Hastings, et al., *Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming*, AMERICAN J. PHYSIOLOGY & CELL PHYSIOLOGY 293:1824–33 (2007), the contents of each of which are hereby incorporated by reference with respect to their teachings regarding cone-and-plate flow devices. An example of such a device is depicted in Figure 1. The device 200 comprises an electronic controller for receiving a set of electronic instructions, a motor 220 operated by the electronic controller, and a shear stress applicator operatively connected to the motor for being driven by the motor. The shear stress applicator can comprise a cone 7, which is attached to the motor, and the cone can be directly driven by the motor. The motor causes the cone to rotate in either direction (clockwise or counterclockwise). The device further comprises a Z-axis micrometer 210 that allows the cone 7 of the device to be raised and lowered.

[00164] The cone-and-plate device accommodates a cell culture container 1, for example a Petri dish (e.g., a 75-mm diameter Petri dish). The cone 7 can be adapted to fit inside the cell culture container. Thus, for example, in a device adapted for use with 75-mm diameter Petri dishes, the cone has a diameter of about 71.4 mm. The cone generally has a shallow cone angle. For example, the angle between the surface of the cone and the surface within the Petri dish is approximately 0.5°–2° (e.g., 1°).

[00165] When the cone 7 of the device 200 is submerged in culture media 3 in the cell culture container 1 and rotated by the motor 220, the cone exerts a rotational stress upon the culture media, and this in turn applies shear stress to cells plated within the cell culture container or to a surface of a porous membrane 11 suspended in the cell culture container. For example, cells 6 can be plated on a first surface of a porous membrane, the cone can be

used to apply a shear stress to the opposing surface of the membrane as depicted in the inset in Figure 1.

[00166] A porous membrane can be suspended in the cell culture container using a cell culture insert 4 that includes a porous membrane 11 and a support 10. The cell culture insert is adapted to fit inside the cell culture container. The cell culture insert suitably has a height that is shorter than the height of the cell culture container, such that when the cell culture insert is placed into the cell culture container, the support portion of the cell culture insert contacts the perimeter of the cell culture container and holds the porous membrane in a suspended position within the cell culture container. For example, the insert can have a rim that extends around the perimeter of the insert, wherein the rim of the insert then contacts the perimeter of the cell culture container to suspend the insert in the cell culture container. Cell culture inserts in a variety of sizes are commercially available from a number of manufacturers (e.g., Corning, which manufactures TRANSWELL inserts; Millicell; and ThinCert). The porous membranes can be made of any suitable porous material (e.g., polyester, polycarbonate, collagen-coated polytetrafluoroethylene (PTFE), or polyethylene terephthalate (PET)) and can have a variety of thicknesses and pore sizes.

[00167] The cone-and-plate device can also include a base 240 for securely holding the cell culture container. The device can also include clips that mount on the cell culture container dish and secure inflow and outflow tubing, which is used to perfuse the upper and lower volumes, as described further below.

[00168] The flow can be derived from a previously measured hemodynamic pattern, and can be modeled into a set of electronic instructions. The shear stress is based on the set of electronic instructions.

[00169] The flow device can comprise a body adapted for being positioned in the culture medium in the upper volume of the cell culture container and a motor adapted to rotate the body. The body can have a conical surface or a flat surface.

[00170] The flow device can be adapted for positioning the conical or flat surface of the body in the cell culture container and in contact with the culture medium.

[00171] The flow device can comprise an electronic controller for receiving the set of electronic instructions. The motor is operated by the electronic controller. A shear stress applicator operatively connected to the motor is driven by the motor. Preferably, the shear stress applicator comprises a cone or a disc attached to the motor.

[00172] The flow device is used in conjunction with a cell culture container. The cell culture container can include inlets and outlets for perfusing cell culture medium, drugs, compounds, and other components into and out of the cell culture container.

[00173] The inlets and outlets can be secured to the cell culture container by clips. Figure 2 depicts a clip. Each clip 300 is made up of three parts: the main body 301 and two pieces of thin metal tubing 302 and 303 as shown in FIG. 2. The clip 300 can be secured to the side of a cell culture container from the outside by a screw 304. For example, two clips can be attached and tightened to the side of the container from the outside by a screw 304. The main body 301 is made of treated stainless steel metal and angles around the edge of the dish for attachment and access purposes. Two pieces of thin metal tubing (302 and 303) per clip are bent to provide access to the dish for supplying and drawing off media efficiently, without obstructing the cone rotation. A set screw 305 on either side of the main body 301 secures the metal tubing 302, 303 to the main body and holds the metal tubing in place such that it extends to the correct depth within the culture media. Flexible tubing then slides over the metal tubing, which is used to perfuse media into and out of the cell culture container (e.g., from a source bottle to the container via mechanical peristaltic pump).

[00174] Figure 3 shows two clips 300 positioned in a cell culture container 1. In the configuration shown in Figure 3, a porous membrane 11 is suspended in the cell culture container. Figure 3 also depicts the cone 7 of a cone-and-plate flow device and the culture medium 3.

[00175] As the cone of the cone-and-plate device rotates, fluid is transported in a concentric manner within the upper volume of the cell culture container. In addition, the rotation of the cone causes a downward flow of cell culture medium through the porous membrane and through any cells plated on the porous membrane and/or extracellular matrix components deposited on the porous membrane.

Hemodynamic Patterns

[00176] The flow can be derived from a previously measured hemodynamic pattern.

[00177] The hemodynamic pattern can be derived from the vasculature of a tumor.

[00178] The hemodynamic pattern can be derived from at least a portion of a capillary, an arteriole, an artery, a venule, or a vein.

[00179] The hemodynamic pattern can be derived from at least a portion of an organ. For example, the hemodynamic pattern can be derived from a liver, a kidney, a lung, a brain, a pancreas, a spleen, a large intestine, a small intestine, a heart, a skeletal muscle, an eye, a tongue, a reproductive organ, or an umbilical cord.

[00180] The hemodynamic pattern can be derived from analysis of ultrasound data.

[00181] The hemodynamic pattern can be derived from analysis of magnetic resonance imaging (MRI) data.

[00182] The flow or the hemodynamic pattern can be time-variant.

[00183] The flow or the hemodynamic pattern can be derived from an animal, such as a genetically modified animal or a human. Preferably, the hemodynamic pattern is derived from a human.

[00184] For example, Figure 4A shows a Doppler sonography image of the central bronchial artery in a patient diagnosed with a pulmonary lesion. The mono-phasic low-impedance flow signal is indicative of a malignant lesion. Figure 4B depicts wall shear stress calculations (dynes/cm^2) of the Doppler flow signal of a human pulmonary lesion. Figure 4C provides a schematic illustration of an exemplary arterial blood supply in a pulmonary lesion.

[00185] The shear stress applied upon the at least one tumor cell type can be about 0.1 dynes/cm^2 to about 200 dynes/cm^2. For example, the shear stress applied upon the at least one tumor cell type can be about 0.1 dynes/cm^2 to about 100 dynes/cm^2.

[00186] The shear stress can be applied at a rate of about 1 sec^{-1} to about 1000 sec^{-1}.

Exemplary Methods for Mimicking a Tumor Microenvironment *In Vitro*

[00187] Figures 5 through 10 are schematic diagrams illustrating exemplary methods for mimicking a tumor microenvironment *in vitro*. In each of Figures 5–10, a cell culture container 1 contains a culture medium 3.

[00188] In Figures 5–8, the cell culture container also contains a porous membrane 11. The porous membrane is suspended in the cell culture container such that a first surface of the porous membrane is proximal and in spaced relation to the bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume 23 comprising the tumor cells 5 and an upper volume 25 comprising a second surface of the porous membrane. The porous membrane shown in Figures 5–8 can be a porous membrane of a cell culture insert adapted to fit inside the cell culture container.

[00189] In Figures 5–8, at least one extracellular matrix component (ECM) 9 is present on the first surface of the porous membrane. The extracellular matrix component can be endogenously produced by cells plated within the cell culture container. Alternatively, exogenous extracellular matrix can be deposited on the first surface of the porous membrane by any of the methods described herein. The extracellular matrix can include both endogenous extracellular matrix produced by cells plated within the cell culture container and exogenously added extracellular matrix.

[00190] In Figures 5–8, tumor cells 5 are also present on the first surface of the porous membrane and are substantially surrounded by the ECM. In Figures 6 and 7, both tumor cells 5 and stromal fibroblasts 15 are also present on the first surface of the porous membrane and are substantially surrounded by the ECM. In Figure 6, the stromal fibroblasts and tumor cells are plated sequentially, with the stromal fibroblasts being plated first, and the tumor cells being plated on the plated stromal fibroblasts. In Figure 7, the stromal fibroblasts and tumor cells are mixed together with one another prior to plating.

[00191] In each of Figures 5–8, endothelial cells 13 are plated on the second surface of the porous membrane. In Figure 8, stromal fibroblasts 15 are also plated on the second surface of the porous membrane. In Figure 8, the stromal fibroblasts and endothelial cells are plated sequentially, with the stromal fibroblasts being plated first on the second surface of the porous membrane, and the endothelial cells being plated on the plated stromal fibroblasts. Alternatively, although not depicted, the stromal fibroblasts and endothelial cells can be mixed together with one another prior to plating.

[00192] Figures 9 and 10 depict methods that use two porous membranes, a first porous membrane and a second porous membrane. In Figure 9, stromal fibroblasts 15 are plated on a first surface of a first porous membrane 11. The porous membrane is suspended in the cell culture container such that a first surface of the first porous membrane is proximal and in spaced relation to the bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume 23 comprising the tumor cells 5 and an upper volume 25 comprising a second surface of the porous membrane. The first porous membrane 11 can be a porous membrane of a cell culture insert adapted to fit inside the cell culture container. A second porous membrane 12 is placed on the plated stromal cell type, such that a first surface of the second porous membrane contacts the plated stromal fibroblasts. ECM 9 is present on the second surface of the second porous membrane. The ECM can be endogenously produced by cells plated within the cell culture container. Alternatively, ECM

can be deposited on the second surface of the second porous membrane by any of the methods described herein. The ECM can include both endogenous ECM produced by cells plated within the cell culture container and exogenously added ECM. Tumor cells 5 are also present on the second surface of the second porous membrane and are substantially surrounded by the ECM. Endothelial cells 13 are plated on the second surface of the first porous membrane.

[00193] In Figure 10, ECM 9 is present on the first surface of a first porous membrane 11. The porous membrane is suspended in the cell culture container such that a first surface of the porous membrane is proximal and in spaced relation to the bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume 23 comprising the tumor cells 5 and an upper volume 25 comprising a second surface of the porous membrane. The first porous membrane can be a porous membrane of a cell culture insert adapted to fit inside the cell culture container. The ECM can be endogenously produced by cells plated within the cell culture container. Alternatively, ECM can be deposited on the first surface of the first porous membrane by any of the methods described herein. The ECM can include both endogenous ECM produced by cells plated within the cell culture container and exogenously added ECM. Tumor cells 5 are also present on the first surface of the first porous membrane and are substantially surrounded by the ECM. Stromal fibroblasts 15 are plated on a second surface of the first porous membrane. A second porous membrane 12 is placed on the plated stromal fibroblasts such that a first surface of the second porous membrane contacts the plated stromal fibroblasts. Endothelial cells 13 are plated on the second surface of the second porous membrane.

[00194] Figures 5–10 also show inlets 17 and outlets 19 that can be used for perfusing cell culture medium, drugs, compounds, and other components into and out of the cell culture container.

[00195] The cone 7 of a cone-and-plate flow device is also shown in Figures 5–10. The cone induces concentric flow of the culture medium within the upper volume of the cell culture container, as represented by the dotted circular arrow. The flow of the medium in turn applies a shear stress upon the endothelial cells. The dotted arrows pointing downwards towards the bottom of the cell culture container represent the downward transport of medium, drugs, and other components through the porous membrane that occurs upon application of the shear stress and perfusion of culture medium into and out of the cell culture container.

Methods for Mimicking Tumor Metastasis

[00196] The present invention further relates to methods for mimicking tumor metastasis. The methods for mimicking tumor metastasis include methods for mimicking tumor metastasis *in vitro*, and methods for mimicking tumor metastasis in an animal.

Methods for Mimicking Tumor Metastasis In Vitro

[00197] A method for mimicking tumor metastasis *in vitro* is provided. The method comprises introducing cells of at least one tumor cell type cultured according to any one of the methods described above into an *in vitro* system that models an organ or tissue. For example, the *in vitro* system that models the organ or tissue can be an *in vitro* system that models the liver, pancreas, bone, lung, blood vessels, the lymphatic system, brain, muscle, bladder, kidney, intestine, colon, gall bladder, skin, or bone.

[00198] *In vitro* systems that model the liver are described in U.S. Patent Application Publication No. US 2013/0309677 and PCT Publication No. 2013/0158939, the contents of both of which are hereby incorporated by reference in their entirety and which are described herein in the section entitled "*In vitro* systems that model the liver," Examples12-14, and Figures 20–43. An *in vitro* system that models the liver described in U.S. Patent Application Publication No. US 2013/0309677 comprises a cell culture container containing a culture medium and a porous membrane, wherein hepatocytes are plated on a first surface of the porous membrane and the porous membrane is suspended in the another cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the container, thereby defining within the container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane. A shear stress is applied upon the second surface of the porous membrane in the upper volume, the shear stress mimicking flow to which the hepatocytes are exposed *in vivo*. The cell culture container further comprises inlets within the portions of the cell culture container defining the upper and lower volumes. The cell culture container can also comprise outlets within the portions of the cell culture container defining the upper and lower volumes.

[00199] Thus, in the method for mimicking tumor metastasis *in vitro*, the *in vitro* system that models the liver can comprise another cell culture container comprising a culture medium and a porous membrane. Hepatocytes are plated on a first surface of the porous membrane, and the porous membrane is suspended in the another cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the container,

thereby defining within the container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane. A shear stress is applied upon the second surface of the porous membrane in the upper volume, the shear stress mimicking flow to which the hepatocytes are exposed *in vivo*. The another cell culture container further comprises inlets within the portions of the cell culture container defining the upper and lower volumes.

[00200] In the *in vitro* system that models the liver described in U.S. Patent Application Publication No. US 2013/0309677 and PCT Publication No. 2013/0158939, at least one extracellular matrix component (e.g., a collagen) can be plated on the first surface of the porous membrane, and the hepatocytes can be plated on the at least one extracellular matrix component. An additional layer of at least one extracellular matrix component can be deposited on top of the hepatocytes, such that the at least one extracellular matrix component substantially surrounds the hepatocytes.

[00201] In the *in vitro* system that models the liver described in U.S. Patent Application Publication No. US 2013/0309677 and PCT Publication No. 2013/0158939, sinusoidal endothelial cells can be plated on the second surface of the porous membrane. Additional non-parenchymal cell types, such as hepatic stellate cells, Kupffer cells, or a combination thereof, can also be plated on the first or second surface of the porous membrane.

[00202] Figure 11 provides a schematic diagram illustrating an exemplary *in vitro* system that models the liver. A cell culture container 1' contains a culture medium 3'. The cell culture container also contains a porous membrane 11'. The porous membrane is suspended in the cell culture container such that a first surface of the porous membrane is proximal and in spaced relation to the bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume 23' comprising hepatocytes and an upper volume 25' comprising a second surface of the porous membrane. The porous membrane can be a porous membrane of a cell culture insert adapted to fit inside the cell culture container. An extracellular matrix component (ECM) 9' is present on the first surface of the porous membrane. Hepatocytes 100 and Kupffer cells 120 are also present on the first surface of the porous membrane and are substantially surrounded by the ECM. The ECM can be endogenously produced by cells plated within the cell culture container. Alternatively, exogenous ECM can be deposited on the first surface of the porous membrane by any of the methods described herein. The ECM can include both endogenous ECM produced by cells

plated within the cell culture container and exogenously added ECM. Sinusoidal endothelial cells 110 are plated on the second surface of the porous membrane.

[00203] Figure 11 also shows inlets 17' and outlets 19' that can be used for perfusing culture medium, drugs, compounds, cells, and other components into and out of the cell culture container.

[00204] Figure 11 also depicts the cone 7' of a cone-and-plate flow device. The cone induces flow of the culture medium, as represented by the dotted circular arrow in the upper volume. The flow of the medium in turn applies a shear stress upon the endothelial cells. The dotted arrows pointing downwards towards the bottom of the cell culture container represent the transport of medium, drugs, and other components that occurs upon application of the shear stress and perfusion of culture medium into and out of the cell culture container.

[00205] In the method for mimicking tumor metastasis *in vitro*, the cells of the at least one tumor cell type can be introduced into the *in vitro* system that models the liver by transferring the cells of the at least one tumor cell type into the lower volume or the upper volume of the *in vitro* system that models the liver. When the cells of the at least one tumor cell type are introduced into the upper volume of the *in vitro* system that models the liver, the method can further comprise assessing migration of the cells of the at least one tumor cell type into the lower volume of the *in vitro* system that models the liver. Assessing migration of the tumor cells from the upper volume to the lower volume can be achieved by fixing the cells in the upper and lower chamber and performing microscopic imaging of the cells in the upper and lower volumes, and/or by sorting the cells in the upper or lower volumes where the tumor cells are labeled with a molecular tracer (e.g., a radiolabelled probe, a fluorescent protein, or colorometric tracer).

[00206] The transferring can comprise manually transferring the cells of the at least one tumor cell type into the lower volume or the upper volume of the *in vitro* system that models the liver. For example, the cells of the least one tumor cell type can be removed from the surface within the cell culture container or the porous membrane by trypsinization or by enzymatic digestion of the ECM, if present. The cells of the at least one tumor cell type can then be transferred to the upper or lower volume of the *in vitro* system that models the liver using a pipette. Alternatively, culture medium from the upper or lower volume of the cell culture container containing the at least one tumor cell type can be pipetted into the upper or lower volume of the *in vitro* system that models the liver.

[00207] Alternatively, the transferring can comprise pumping cell culture medium out of the upper or lower volume of the cell culture container containing the at least one tumor cell type and into the upper or lower volume of the cell culture container of the *in vitro* system that models the liver. This mimics the seeding of distal organs by tumor cells *in vitro*. Such methods are illustrated in Figures 12A–D. In Figure 12A, for example, tubing 21 is used to connect to an outlet 19 within the lower volume 23 of the cell culture container 1 comprising the at least one tumor cell type 5 to an inlet 17' in the lower volume 23' of the cell culture container 1' of the *in vitro* system that models the liver. The culture medium can be pumped through the tubing to transfer the cells of the at least one tumor cell type into the lower volume of the *in vitro* system that models the liver. The methods depicted in Figures 12B, C, and D are similar, but involve pumping culture medium from the lower volume 23 of the cell culture container 1 that contains the at least one tumor cell type 5 into the upper volume 25' of the cell culture container 1' of the *in vitro* system that models the liver (Fig. 12B), pumping culture medium from the upper volume 25 of the cell culture container 1 that contains the at least one tumor cell type 5 into the lower volume 23' of the cell culture container 1' of the *in vitro* system that models the liver (Fig. 12C), or pumping culture medium from the upper volume 25 of the cell culture container 1 that contains the at least one tumor cell type 5 into the upper volume 25' of the cell culture container 1' of the *in vitro* system that models the liver (Fig. 12D).

[00208] Thus, the cell culture container comprising the at least one tumor cell type can further comprise an outlet within the portion of the cell culture container defining the lower volume and containing the at least one tumor cell type. The outlet is connected to an inlet in the another cell culture container of the *in vitro* system that models the liver. The transferring comprises pumping the culture medium out of the lower volume of the cell culture container comprising the at least one tumor cell type and into the upper or lower volume of the another cell culture container of the *in vitro* system that models the liver.

[00209] Alternatively, the cell culture container comprising the at least one tumor cell type can further comprise an outlet within the portion of the cell culture container defining the upper volume. The outlet is connected to an inlet in the another cell culture container of the *in vitro* system that models the liver. The transferring comprises pumping the culture medium out of the upper volume of the cell culture container and into the upper or lower volume of the another cell culture container of the *in vitro* system that models the liver.

[00210] The present invention further relates to another method for mimicking tumor metastasis *in vitro*. The method comprises adding a culture medium to a cell culture container and plating at least one cell type on a first surface of a porous membrane within the cell culture container, wherein the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one cell type and an upper volume comprising a second surface of the porous membrane. A shear stress is indirectly applied upon the at least one cell type, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the cells are indirectly exposed *in vivo*. Tumor cells derived from a human or a humanized animal are introduced into the upper volume or the lower volume.

[00211] The at least one cell type can comprise hepatocytes or smooth muscle cells.

[00212] The method can further comprise plating a second cell type on the second surface of the porous membrane. The second cell type can comprise endothelial cells.

[00213] Where the tumor cells are derived from a humanized animal, the humanized animal is suitably a humanized mouse, for example, a non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse, a NOD/Shi-scid/IL-2Rγnull (NOG) mouse, or a NOD SCID IL-2Rγ knockout (NSG) mouse.

[00214] The present invention further relates to an *in vitro* method of testing a drug or a compound for an effect on tumor metastasis. The method comprises mimicking tumor metastasis *in vitro* by any of the methods described above, and adding a drug or a compound to the culture medium. A change in the cells of the at least one tumor cell type in the *in vitro* system that models the organ or tissue, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on tumor metastasis.

[00215] To confirm *in vitro* mimicking of tumor metastasis, a change in the level or localization of a marker of tumor metastasis can be compared between the method of the invention and the same method in the absence of the application of the shear stress. The level or localization of the marker in the at least one tumor cell type upon application of the shear stress is compared to the level or localization of the marker in the at least one tumor cell type in the absence of the application of the shear stress. Alternatively, the level of marker in the culture medium upon application of the shear stress is compared to the level of the marker in the culture medium upon the absence of the application of shear stress. For example, if a

marker is known to be associated with tumor metastasis, and its concentration is known to increase in the serum during metastasis *in vivo*, an increase in the level of the marker in the culture medium of the method of the invention with application of shear stress as compared to the level of the marker in the culture medium in the absence of the application of the shear stress confirms that tumor metastasis is mimicked by the *in vitro* method of the invention.

Methods for mimicking tumor metastasis in an animal

[00216] The present invention also provides a method for mimicking tumor metastasis. The method comprises introducing cells of the at least one tumor cell type cultured according to any of the methods described above into an animal. The animal can be a mammal, for example a mouse, rat, guinea pig, hamster rabbit, cat, dog, monkey, cow, pig, horse, goat, or sheep. Alternatively, the animal can be a bird or a fish.

[00217] Where the animal is a mouse, the mouse is suitably a humanized mouse, and the at least one tumor cell type comprises a human tumor cell type. The humanized mouse can be a non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse, a NOD/Shi-scid/IL-2Rγnull (NOG) mouse, or a NOD SCID IL-2Rγ knockout (NSG) mouse.

Personalized Medicine

[00218] The present invention provides a method for selecting a chemotherapy regimen to be administered to a subject having a tumor. The method comprises testing a drug or a compound *in vitro* for an effect on a tumor or testing a drug or a compound for an *in vitro* effect on tumor metastasis according to any of the methods described herein. The at least one tumor cell type comprises tumor cells derived from the subject's tumor. The method further comprises determining whether to administer the drug or the compound to the subject based on the results of the *in vitro* testing.

[00219] The method can further comprise selecting a dose of the drug or the compound to be administered to the subject based on the results of the *in vitro* testing. The dose selected will be a dose that is predicted to be both therapeutic and safe in the subject based on the results of the *in vitro* testing.

[00220] The method can further comprise selecting a rate of administration of the drug or the compound to be administered to the subject based on the results of the *in vitro* testing. The rate selected will be a rate that is predicted to be both therapeutic and safe in the subject based on the results of the *in vitro* testing.

Drugs and Compounds

[00221] In any of the *in vitro* methods of testing a drug or a compound for an effect on a tumor or for an effect on tumor metastasis described herein, the at least one tumor cell type can be exposed to the drug or the compound directly or indirectly. The at least one tumor cell type can be directly exposed to the drug or compound by adding the drug or compound to cell culture medium containing or contacting the at least one tumor cell type. For example, where the at least one tumor cell type is plated on a first surface of a porous membrane, and the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one tumor cell type and an upper volume comprising a second surface of the porous membrane, the at least one tumor cell type can be directly exposed to the drug or compound by adding the drug or compound to the cell culture medium in the lower volume. Adding the drug or compound to the upper volume can also result in directly exposing the at least one tumor cell type to the drug or compound, for example where the drug or compound is small enough to diffuse through the pores of the membrane or where the tumor cells have migrated into the upper volume. Alternatively, the at least one tumor cell type can be indirectly exposed to the drug or compound. For example, the at least one tumor cell type would be indirectly exposed to the drug or the compound when the drug or the compound is added to the upper volume and the drug or compound does not diffuse through the pores of the membrane, but exerts an effect on endothelial cells plated on the second surface of the porous membrane which in turn causes the endothelial cells to exert an effect on the at least one tumor cell type (e.g., secretion of a factor by the endothelial cells that diffuses through the porous membrane and has an effect on the at least one tumor cell type, or physical interaction of the endothelial cells with the at least one tumor cell type through the pores of the porous membrane).

[00222] The concentration of the drug or compound in the culture medium can be within the concentration range of the drug or the compound that achieves the effect *in vivo*. For example, the concentration of the drug or the compound in the culture medium can be within the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound (e.g., the *in vivo* therapeutic plasma C_{max} for the drug or the compound). The concentration of the drug or the compound in the culture medium can be approximately the same as the *in vivo*

therapeutic C_{max} for the drug or the compound (e.g., the *in vivo* therapeutic plasma C_{max} for the drug or the compound).

[00223] Alternatively, the concentration of the drug or the compound in the culture medium can be lower than the concentration range of the drug or the compound that achieves the effect *in vivo*. This mimics the lower degree of drug penetration that is observed in many solid tumors *in vivo*. For example, the concentration of the drug or the compound in the culture medium can be about 2-fold to about 20-fold lower, about 5-fold to about 15-fold lower, or about 10-fold lower than the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound (e.g., the *in vivo* therapeutic plasma C_{max} for the drug or the compound).

[00224] The effect of the drug or the compound can comprise a toxic effect, a protective effect, a pathologic effect, a disease-promoting effect, an inflammatory effect, an oxidative effect, an endoplasmic reticulum stress effect, a mitochondrial stress effect, an apoptotic effect, a necrotic effect, an autophagic effect, an immunogenic cell death effect, a ferroptotic effect, a remodeling effect, a proliferative effect, an effect on angiogenesis, an effect on the activity of a protein, or an effect on the expression of a gene. The term "proliferative effect" encompasses both stimulation of proliferation and inhibition of proliferation. Similarly, the effect on angiogenesis encompasses both stimulation of angiogenesis and inhibition of angiogenesis.

[00225] Where the effect comprises the effect on the activity of a protein, the effect can comprise inhibition of the protein or activation of the protein.

[00226] Where the effect comprises the effect on the expression of a gene, the effect can comprise an increase in the expression of the gene or a decrease in the expression of the gene.

[00227] The *in vitro* methods of testing a drug or a compound for an effect on a tumor or on tumor metastasis can be used to screen candidate molecules for anti-cancer activity.

[00228] The *in vitro* methods of testing a drug or a compound for an effect on a tumor or on tumor metastasis can also be used to test drugs or compounds known or suspected to have anti-cancer activity.

[00229] The drug or compound can be capable of inhibiting, activating, or altering the function of proteins or genes in the at least one cell type.

[00230] The drug can comprise an anti-cancer agent. Anti-cancer agents include, for example, alkylating agents, anti-metabolites, anti-tumor antibiotics, topoisomerase inhibitors, corticosteroids, anti-microtubule agents, kinase inhibitors, pathway inhibitors, differentiating agents, hormone therapies, immunotherapies, L-asparaginase, chelating agents, ATP mimetics, biologic medical products, and combinations thereof.

[00231] When the anti-cancer agent comprises the alkylating agent, the alkylating agent can comprise altretamine, bendamustine, busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, ifosfamide, lomustine, mechlorethamine, melphalan, oxalaplatin, palifosamide, streptozocin, temozolomide, thiotepa, or a combination thereof.

[00232] When the anti-cancer agent comprises the anti-metabolite, the antimetabolite can comprise azathioprine, capecitabine, cladribine, clofarabine, cytarabine, floxuridine, fludarabine, fluorouracil, gemcitabine, hydroxyurea, mercaptopurine, methotrexate, nelarabine, pemetrexed, pentostatin, pralatrexate, raltitrexed, thioguanine, or a combination thereof.

[00233] When the anti-cancer agent comprises the anti-tumor antibiotic, the anti-tumor antibiotic can comprise bleomycin, dactinomycin, mitomycin, plicamycin, rifampicin, or a combination thereof.

[00234] When the anti-cancer agent comprises the topoisomerase inhibitor, the topoisomerase inhibitor can comprise amsacrine, topotecan, irinotecan , etoposide, teniposide, mitoxantrone, etirinotecan, camptothecin, daunorubicin, doxorubicin, epirubicin, idarubicin, valrubicin, amonafide, or a combination thereof.

[00235] When the anti-cancer agent comprises the corticosteroid, the corticosteroid can comprise prednisone, methylprednisolone, dexamethasone, cortisol sodium succinate, or a combination thereof.

[00236] When the anti-cancer agent comprises the anti-microtubule agent, the anti-microtubule agent can comprise vinblastine, vincristine, vindesine, vinorelbine, paclitaxel, docetaxel, ixabepilone, eribulin mesylate, cabazitaxel, or a combination thereof.

[00237] When the anti-cancer agent comprises the kinase inhibitor, the kinase inhibitor can comprise a small molecule inhibitor of a receptor or non-receptor tyrosine kinase, a serine/threonine-specific kinase inhibitor, or a dual-specificity kinase inhibitor.

[00238] The kinase inhibitor can comprise an epidermal growth factor (EGF) receptor inhibitor, a fibroblast growth factor (FGF) receptor inhibitor, a platelet-derived

growth factor (PDGF) receptor inhibitor, a vascular endothelial growth factor (VEGF) receptor inhibitor, or a rho kinase inhibitor.

[00239] When the kinase inhibitor comprises the small molecule inhibitor of a receptor or non-receptor tyrosine kinase, the small molecule inhibitor of a receptor or non-receptor tyrosine kinase can comprise afatinib, alectinib, alisertib, amuvatinib, apatinib, axitinib, bafetinib, barasertib, baricitinib, bosutinib, brivanib, buparlisib, cabozantinib, canertinib, cenisertib, cobimetinib, crenolanib, crizotinib, dabrafenib, dacomitinib, danusertib, desatinib, dovitinib, epitinib, erlotinib, foretinib, fostamatinib, galunisertib, gefitinib, ibrutinib, imatinib, lapatinib, lenvatinib, lestaurtinib, linifanib, linsitinib, masitinib, momelotinib, motesanib, mubritinib, neratinib, nilotinib, nintedanib, orantinib, pacritinib, pazopanib, pelitinib, pimasertib, ponatinib, poziotinib, quizartinib, refametinib, regorafenib, ruxolitinib, selumetanib, sorafenib, sulfatinib, sunitinib, tandutinib, telatinib, theliatinib, tivantinib, tofacitinib, trametinib, vandetanib, vatalinib, vemurafenib, volasertib, volitinib, or a combination thereof.

[00240] When the kinase inhibitor comprises the serine/threonine-specific kinase inhibitor, the serine/threonine-specific kinase inhibitor can comprise MK2206.

[00241] When the anti-cancer agent comprises the pathway inhibitor, the pathway inhibitor can comprise a B-cell lymphoma 2 (Bcl-2) family inhibitor (e.g., navitoclax, obatoclax, oblimerson, or cinacalcet), a heat shock protein 90 (HSP-90) inhibitor (e.g., tanespimycin, retaspimycin, or ganetespib), a proteasome inhibitor (e.g., bortezomib, carfilzomib, oprozomib, ixazomib, marozomib, or delanzomib), a cyclin-dependent kinase inhibitor (e.g., flavopiridol, alvocidib, dinaciclib, seliciclib, or palbociclib), an inhibitor of poly ADP-ribose polymerase (PARP) (e.g., iniparib, veliparib, olaparib, rucaparib, or niraparib), an inhibitor of the mammalian target of rapamycin (mTOR) (e.g., deforolimus, everolimus, sirolimus, or temsirolimus), an inhibitor of histone deacetylase (HDAC) (e.g., belinostat, entinostat, mocetinostat, panobinostat, romidepsin, or vorinostat), an inhibitor of the hedgehog pathway (e.g., varidegib or vismodegib), a rho kinase inhibitor (e.g., Y27632), or a combination thereof.

[00242] When the anti-cancer agent comprises the differentiating agent, the differentiating agent can comprise a retinoid, tretinoin, bexarotene, arsenic trioxide, or a combination thereof.

[00243] When the anti-cancer agent comprises the hormone therapy, the hormone therapy can comprise a selective androgen-receptor modulator (SARM) (e.g., enobosarm), an

androgen receptor antagonist (e.g., bicalutamide, flutamide, nilutamide, or enzalutamide), a selective estrogen receptor modulator (SERM) (e.g., tamoxifen, toremifene, or raloxifene), an estrogen receptor antagonist (e.g., fulvestrant), a progestin (e.g., megestrol acetate), an estrogen (e.g., estramustine), an aromatase inhibitor (e.g., anastrozole, exemestane, or letrozole), a gonadotropin-releasing hormone (GnRH) agonist or analog (e.g., leuprolide, goserelin, abarelix, degarelix, or triptorelin), ketoconazole, abiraterone, or a combination thereof.

[00244] When the anti-cancer agent comprises the immunotherapy, the immunotherapy can comprise a monoclonal antibody (e.g., rituximab, alemtuzumab, bevacizumab, abagovomab, or etaracizumab), a non-specific immunotherapy or adjuvant (e.g., interleukin-2 (IL-2), interferon-α, interferon-α2b, peginterferon alfa-2b, abatacept, or aldesleukin), an immunomodulating drug (e.g., thalidomide or lenalidomide), a cancer vaccine (e.g., Sipuleucel-T or Bacillus Calmette-Guérin (BCG) vaccine), a targeted immunotherapy (e.g., brentuzimab, cetuximab, ibritumomab, ipilimumab, ofatumumab, panitumumab, pertuzumab, tositmuomab, trastuzumab, tremelimumab, siltuximab, tocilizumab, canakinumab, lirilumab, nivolumab, pidilizumab, or lambrolizumab), or a combination thereof.

[00245] When the anti-cancer agent comprises the chelating agent, the chelating agent can comprise penicillamine, triethylene tetramine dihydrochloride, EDTA, DMSA, deferoxamine mesylate, or batimastat.

[00246] When the anti-cancer agent comprises the biologic medical product, the biologic medical product can comprise a synthetic polysaccharide; a synthetic, partially synthetic or humanized immunoglobulin; or a recombinant therapeutic protein.

[00247] The drug or the compound can comprise a radiocontrast agent, a radio-isotope, a prodrug, an antibody fragment, an antibody, a live cell, a therapeutic drug delivery microsphere, microbead, nanoparticle, gel or cell-impregnated gel, or a combination thereof.

[00248] In any of the *in vitro* methods of testing a drug or a compound for an effect on a tumor or for an effect on tumor metastasis described herein, adding the drug or the compound to the culture medium can comprise adding an antibody-drug conjugate or a modified release dosage form comprising the drug or the compound to the culture medium.

[00249] The modified release dosage form can comprise an oral modified release dosage form.

[00250] The modified release dosage form can a modified release polymer (e.g., hydroxypropylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, ethylcellulose, methylcellulose, carboxymethylcellulose, alginic acid, carrageenan, chitosan, heparin, starch, xanthan gum, polyvinyl alcohol, polyacrylic acid, polyethylene oxide, poloxamers, pluronics, polymethacrylate, polysialic acid, or a combination thereof).

[00251] The method can comprise perfusing the drug or the compound into at least one of the upper volume and the lower volume.

Analysis of the Cell Types and Cell Culture Medium

[00252] In methods of the invention involving comparing a change in the level or localization of a marker of the tumor microenvironment or a marker of tumor metastasis between a method of the invention and the same method in the absence of the application of the shear stress, the marker can comprise a marker of cell proliferation, cell invasion, angiogenesis, tumorigenesis, cell monolayer integrity, endothelial cell barrier function, permeability, inflammation, cell death, apoptosis, necrosis, contraction, cell motility, or a combination thereof.

[00253] The change in the level of a marker can be an increase in the level of the marker in the at least one tumor cell type or the endothelial cells.

[00254] The change in the level of a marker can be a decrease in the level of the marker in the at least one tumor cell type or the endothelial cells.

[00255] The marker can comprise VE-cadherin, E-cadherin, actin, or a combination thereof.

[00256] When the marker comprises a marker of angiogenesis, the marker of angiogenesis can comprise vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, angiopoietin-1 (ANG1), angiopoietin-2 (ANG2), fibroblast growth factor-2 (FGF-2), placental growth factor (PLGF), or a combination thereof.

[00257] When the marker comprises a marker of cell proliferation, the marker of cell proliferation can comprise epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), MKI67, proliferating cell nuclear antigen (PCNA), or a combination thereof.

[00258] When the marker comprises a marker of cell invasion, the marker of cell invasion can comprise vimentin (VIM), cadherin 1 (CDH-1), cadherin 2 (CDH-2), or a combination thereof.

[00259] When the marker comprises a marker of inflammation, the marker of inflammation can comprises interleukin-6 (IL-6), interleukin-8 (IL-8), NF-κB, endothelial nitric oxide synthase (eNOS), Krupple-like factor 2 (KLF2), monocyte chemotactic protein-1 (MCP-1), or a combination thereof.

[00260] The methods described herein can further comprise analyzing the endothelial cells for cell density, monolayer integrity, permeability, or a combination thereof.

[00261] The methods described herein can also further comprise analyzing the morphology of the at least one tumor cell type, the endothelial cells, the at least one stromal cell type, or the one or more additional cell types.

[00262] The methods described herein can also further comprise analyzing the culture medium for cytokine secretion, chemokine secretion, humoral factor secretion, microparticle secretion, growth factor secretion, shedding of a protein from the cellular surface, a metabolite of a compound, an immune cell, nitric oxide secretion, a vasodilator protein, a vasoconstrictive protein, miRNA, a secreted protein, or a secreted biological substance. For example, the culture medium can be analyzed for shedding of a protein from the cellular surface, and the protein comprises a vascular cell adhesion molecule (VCAM), E-selectin, or an intracellular adhesion molecule (ICAM). Alternatively or in addition, the culture medium can be analyzed for nitric oxide secretion by measuring nitrate or nitrite concentration.

[00263] In any of the methods comprising adding a drug or a compound to the culture medium, the method can further comprise analyzing the at least one tumor cell type, the endothelial cells, the at least one stromal cell type for toxicity, or the one or more additional cell types for inflammation, permeability, compatibility, cellular adhesion, cellular remodeling, cellular migration, or phenotypic modulation resulting from the drug or the compound.

[00264] Also, in any of the methods comprising adding a drug or a compound to the culture medium, the method can further comprise comparing at least one of the cell types after applying the shear stress for a period of time wherein the medium includes the drug or the compound to the at least one of the cell types after applying the shear stress for the period of time wherein the medium does not include the drug or the compound, to determine the effect of the drug or compound on the at least one of the cell types.

[00265] Any of the methods described herein can further include identifying a drug target. For example, a drug target can be identified by isolating proteins or nucleic acids from

the at least one tumor cell type directly or indirectly exposed to the drug or compound and performing an appropriate screen to identify potential drug targets. Screening methods include proteomic analysis or phosphorylation screening, mRNA analysis (e.g., next generation RNA sequencing or gene arrays), DNA analysis, DNA methylation screening, and intracellular or extracellular miRNA analysis (e.g., miRNA arrays). Modulation of a signal (e.g., increased or decreased expression of a gene) indicates identification of a candidate drug target.

[00266] Any of the methods described herein can further include identifying a surface protein of the at least one tumor cell type, the at least one stromal cell type, the endothelial cells, or the one or more additional cell types as a target for a drug delivery modality. The drug delivery modality can comprise an antibody-drug conjugate, a nanoparticle (e.g., a lipid nanoparticle), a chemical conjugate (e.g., *N*-Acetylgalactosamine (GalNAc)), or a combination thereof. A protein, antibody, peptide, or nucleic acid molecule (e.g., an RNAi molecule) can be conjugated to or incorporated in the nanoparticle or the chemical conjugate. Surface proteins that are targets for a drug delivery modality can be identified by isolating the cell membrane fraction from tumor cells, stromal cells, endothelial cells, or the one or more additional cell types cultured according to any of the methods for mimicking a tumor microenvironment described herein, screening the cell membrane faction to identify potential targets for a drug delivery modality. Screening methods include proteomic analysis or phosphorylation screening, mRNA analysis (e.g., next generation RNA sequencing or gene arrays), DNA analysis, DNA methylation screening, and intracellular or extracellular miRNA analysis (e.g., miRNA arrays). Modulation of a signal (e.g., increased or decreased expression of a gene) indicates identification of a candidate target for a drug delivery modality.

In vitro Systems that Model the Liver

[00267] As noted above, *in vitro* systems that model the liver are described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939. The *in vitro* systems that model the liver described in these publications can be used in method for mimicking an *in vivo* pathological or physiologic condition. Unlike static models currently used as the standard *in vitro* models by the pharmaceutical and biopharmaceutical industries, the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 apply shear forces to cultured cells and replicate an

in vivo pathological or physiological condition using *in vivo* pathological or physiologic concentrations of various factors. For example, an *in vitro* liver model is described in which hepatocytes can be maintained at *in vivo* physiologic concentrations of insulin and glucose that are significantly decreased as compared to the concentrations used in the standard static model. When higher concentrations of insulin and glucose are used in such a model, the hepatocytes exhibit numerous hallmarks of fatty liver disease.

[00268] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 describe a method for mimicking a pathological condition *in vitro* (e.g., a pathological condition of the liver). The method comprises adding a culture media to a cell culture container, adding at least one factor to the culture media, plating at least one cell type on at least one surface within the cell culture container, and applying a shear force upon the at least one plated cell type. The shear force results from flow of the culture media induced by a flow device. The flow mimics flow to which the at least one cell type is exposed *in vivo* in the pathological condition.

[00269] The concentration of the factor in the culture media can be within the *in vivo* concentration range of the factor observed in the pathological condition. Alternatively, the concentration of the factor in the culture media can be within the concentration range of the factor that would result *in vivo* from administration of a drug or a compound.

[00270] To confirm that the *in vivo* pathological condition is mimicked, a change in a level of a marker of the pathological condition can be compared between the method and the same method in the absence of application of the shear force. The level of the marker in the at least one plated cell type or in the culture media upon application of the shear force is compared to the level of the marker in the at least one plated cell type or in the culture media in the absence of application of the shear force. For example, if a marker is known to be associated with a pathological condition and its concentration is known to increase in the serum when the condition is present *in vivo*, an increase in the level of the marker in the culture media of the method with application of the shear force as compared to the level of the marker in the culture media in the absence of application of the shear force confirms that the *in vivo* pathological condition is mimicked by the *in vitro* method.

[00271] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe an *in vitro* method of testing a drug or a compound for an effect on a pathological condition. The method comprises mimicking the pathological condition, adding a drug or a compound to the culture media, and applying the

shear force upon the at least one plated cell type exposed to the drug or the compound. A change in the at least one plated cell type, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on the pathological condition.

[00272] In this *in vitro* method of testing a drug or compound, the pathological condition can be mimicked by the *in vitro* method of mimicking a pathological condition as described above.

[00273] The pathological condition of the *in vitro* method of testing a drug or compound can also be mimicked by plating primary cells or immortalized cells from a subject or subjects having the pathological condition, and culturing the cells in cell culture media.

[00274] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe a method of mimicking a physiologic condition *in vitro* (e.g., a healthy liver). The method comprises adding a culture media to a cell culture container, adding at least one factor to the culture media, plating at least one cell type on at least one surface within the cell culture container, and applying a shear force upon the at least one plated cell type. The shear force results from flow of the culture media induced by a flow device. The flow mimics flow to which the at least one cell type is exposed *in vivo* in the physiologic condition.

[00275] The concentration of the factor in the culture media can be within the *in vivo* concentration range of the factor observed in the physiologic condition. Alternatively, the concentration of the factor in the culture media can be within the concentration range of the factor that would result *in vivo* from administration of a drug or a compound.

[00276] To confirm that the *in vivo* physiologic condition is mimicked, a change in a level of a marker of the physiologic condition can be compared between the method and the same method in the absence of application of the shear force. The level of the marker in the at least one plated cell type or in the culture media upon application of the shear force is compared to the level of the marker in the at least one plated cell type or in the culture media in the absence of application of the shear force. For example, if a marker is known to be associated with a physiologic condition and its concentration is known to increase in the serum when the condition is present *in vivo*, an increase in the level of the marker in the culture media of the method with application of the shear force as compared to the level of the marker in the culture media in the absence of application of the shear force confirms that the *in vivo* physiologic condition is mimicked by the *in vitro* method.

[00277] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe an *in vitro* method of testing a drug or a compound for an effect on a physiologic condition. The method comprises mimicking the physiologic condition, adding a drug or a compound to the culture media, and applying the shear force upon the at least one plated cell type exposed to the drug or the compound. A change in the at least one plated cell type, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on the physiologic condition.

[00278] In this *in vitro* method of testing a drug or compound, the physiologic condition can be mimicked by the *in vitro* method of mimicking a physiologic condition as described above.

[00279] The physiologic condition of this *in vitro* method of testing a drug or compound can also be mimicked by plating primary cells or immortalized cells, and culturing the cells in cell culture media.

[00280] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe an *in vitro* method of testing a drug or a compound for an effect (e.g., an effect on a pathological condition of the liver or on a healthy liver). The method comprises adding a culture media to a cell culture container, plating at least one cell type on at least one surface within the cell culture container, adding a drug or a compound to the culture media, and applying a shear force upon the at least one plated cell type exposed to the drug or the compound. The concentration of the drug or the compound in the culture media is within the concentration range of the drug or the compound that achieves the effect *in vivo*. The shear force results from flow of the culture media induced by a flow device. The flow mimics flow to which the at least one cell type is exposed *in vivo*. A change in the at least one plated cell type, in the presence of the drug or the compound, indicates that the drug or the compound has the effect.

[00281] The effect can be an effect on a pathological condition (e.g., a pathological condition of the liver). Alternatively, the effect can be an effect on a physiologic condition (e.g., a healthy liver.

[00282] In any of the above methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939, the method can further comprise analyzing the cell culture media for cytokine secretion, chemokine secretion, humoral factor secretion, microparticle secretion, growth factor secretion, shedding of a protein from the cellular surface, a metabolite of a compound, an immune cell, nitric

oxide secretion, a vasodilator protein, a vasoconstrictive protein, miRNA, a secreted protein, or a secreted biological substance. The cell culture media can be analyzed for nitric oxide secretion by measuring nitrate or nitrite concentration.

[00283] In any of the above methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939, the method can further comprise the step of culturing the cell type or cell types.

[00284] In any of the above methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 wherein a drug or compound has been added to the culture media, the method can further comprise the step of comparing at least one of the cell types after applying the shear force for a period of time wherein the media includes the drug or the compound to the at least one of the cell types after applying the shear force for the period of time wherein the media does not include the drug or the compound, to determine the effect of the drug or compound on the at least one of the cell types.

[00285] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 describe methods wherein drugs or compounds are tested for an effect on a healthy liver. In such methods, the factors comprise insulin and glucose, hepatocytes are plated on the surface within the cell culture container, and the shear force is applied indirectly to the plated hepatocytes.

[00286] For example, the hepatocytes can be plated on a first surface of a porous membrane. The porous membrane is then suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume and an upper volume. The lower volume comprises the hepatocytes and the upper volume comprises a second surface of the porous membrane. The shear force is applied to the second surface of the porous membrane in the upper volume of the container.

[00287] In the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939, use of a porous membrane suspended in the cell culture container is preferred in plating the cells. When shear force is applied to plated cells or to the surface of the porous membrane (e.g., when the shear is applied on a surface of the membrane absent plated cells), the shear force can enable the cell culture media to perfuse from the upper volume to the lower volume. Such perfusion favorably impacts transport of factors from the upper volume to the lower volume, or vice versa.

[00288] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 describe methods of mimicking a pathological or physiologic condition of the liver *in vitro*. The method comprises adding a culture media to a cell culture container, adding at least one factor to the culture media, plating at least one hepatic cell type on at least one surface within the cell culture container, and applying a shear force upon the at least one plated hepatic cell type. The shear force results from flow of the culture media induced by a flow device. The flow mimics flow to which the at least one hepatic cell type is exposed *in vivo* in the pathological or physiologic condition.

[00289] In this method, the concentration of the factor in the culture media for mimicking the pathological condition can be within the *in vivo* concentration range of the factor observed in the pathological condition. Alternatively, in this method, the concentration of the factor in the culture media for mimicking the pathological condition can be within the concentration range of the factor that would result *in vivo* from administration of a drug or a compound. As a further alternative, in this method, the concentration of the factor in the culture media for mimicking the pathological condition can be capable of maintaining the mimicked pathological condition *in vitro* for a period of time under the shear force, the same concentration of factor being incapable of maintaining the mimicked pathological condition *in vitro* for the period of time in the absence of the shear force.

[00290] In this method, the concentration of the factor in the culture media for mimicking the physiologic condition can be within the *in vivo* concentration range of the factor observed in the physiologic condition. Alternatively, in this method, the concentration of the factor in the culture media for mimicking the physiologic condition can be within the concentration range of the factor that would result *in vivo* from administration of a drug or a compound. As a further alternative, in this method, the concentration of the factor in the culture media for mimicking the physiologic condition can be capable of maintaining the mimicked physiologic condition *in vitro* for a period of time under the shear force, the same concentration of factor being incapable of maintaining the mimicked physiologic condition *in vitro* for the period of time in the absence of the shear force.

[00291] In this method, a change in a level of a marker of the pathological or physiologic condition in the at least one plated hepatic cell type or in the culture media upon application of the shear force, as compared to the level of the marker in the at least one plated hepatic cell type or in the culture media in the absence of application of the shear force confirms mimicking of the pathological or physiologic condition.

[00292] Alternatively, in this method, the at least one plated hepatic cell type can comprise hepatocytes, and responsiveness to glucagon, insulin, or a glucose substrate in the hepatocytes confirms mimicking of the physiologic condition. The glucose substrate can be, for example, glycerol, lactate, pyruvate, or combinations thereof (e.g., a combination of lactate and pyruvate).

[00293] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe an *in vitro* method of testing a drug or a compound for an effect on a pathological or physiological condition (e.g., a pathological or physiological condition of the liver). The method comprises mimicking the pathological or physiological condition, adding a drug or a compound to the culture media, and applying the shear force upon at least one plated hepatic cell type exposed to the drug or the compound. A change in the at least one plated hepatic cell type, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on the pathological or physiological condition.

[00294] In this *in vitro* method of testing a drug or compound, the pathological condition can be mimicked by the *in vitro* method of mimicking a pathological or physiological condition as described directly above.

[00295] The pathological or physiological condition of the *in vitro* method of testing a drug or compound can also be mimicked by plating primary cells or immortalized cells from a subject or subjects having the pathological condition, and culturing the cells in cell culture media.

[00296] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe a method of mimicking a pathological or physiologic condition of the liver *in vitro*. The method comprises adding a culture media to a cell culture container, depositing at least one extracellular matrix component on a surface within the cell culture container, plating hepatocytes on the at least one extracellular matrix component, and indirectly applying a shear force upon the at least one extracellular matrix component and the hepatocytes. The shear force results from flow of the culture media induced by a flow device. The flow mimics flow to which the hepatocytes are exposed *in vivo* in the pathological or physiologic condition.

[00297] In methods in which hepatic cells are plated on a porous membrane, at least one extracellular matrix component can be plated on a first surface of the porous membrane and the hepatic cells can subsequently be plated on the at least one extracellular

matrix component. Optionally, nonparenchymal hepatic cells (e.g., sinusoidal endothelial cells) can be plated on the second surface of the porous membrane, and the shear stress applied to the nonparenchymal hepatic cells.

[00298] In the methods involving the deposition of an extracellular matrix component, for example, the at least one extracellular matrix component can be deposited on a first surface of a porous membrane. The hepatic cell type (e.g., hepatocytes) is subsequently plated on the at least one extracellular matrix component. The porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume and an upper volume. The lower volume comprises at least one extracellular matrix component and the hepatic cell type (e.g., hepatocytes), and the upper volume comprises a second surface of the porous membrane. The shear force is applied to the second surface of the porous membrane in the upper volume of the container. Optionally, nonparenchymal hepatic cells (e.g., sinusoidal endothelial cells) can be plated on the second surface of the porous membrane, and the shear stress applied to the nonparenchymal hepatic cells.

[00299] U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 also describe another method of mimicking a pathological or physiologic condition of the liver *in vitro*. The method comprises adding a culture media to a cell culture container, and plating hepatocytes on a first surface of a porous membrane. The porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the container, thereby defining within the container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane. A shear force is applied upon the second surface of the porous membrane in the upper volume of the container, the shear force resulting from flow of the culture media induced by a flow device. The flow mimics flow to which the hepatocytes are exposed *in vivo* in the pathological or physiologic condition. The flow device comprises a body adapted for being positioned in the culture media in the upper volume of the container and a motor adapted to rotate the body. Preferably, the body has a conical surface. It is also preferred that the flow device is adapted for positioning the conical surface of the body in the container and in contact with the cell culture media.

[00300] This method can further comprise plating nonparenchymal hepatic cells on the second surface of the porous membrane, wherein the shear stress is applied to the

nonparenchymal hepatic cells. The nonparenchymal hepatic cells can comprise sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells, or combinations thereof.

[00301] In the *in vitro* methods for mimicking a pathological or physiologic condition of the liver, a change in a level of a marker of the pathological or physiologic condition can be compared in the method to the same method in the absence of application of the shear force. A change in the level of the marker in any of the hepatic cells or in the culture media upon application of the shear force as compared to the level of the marker in the hepatic cells or in the culture media in the absence of application of the shear force confirms mimicking of the pathological or physiologic condition. For example, a change in the level of the marker in the hepatocytes or nonparenchymal hepatic cells or in the culture media upon application of the shear force as compared to the level of the marker in the hepatocytes or nonparenchymal hepatic cells or in the culture media in the absence of application of the shear force confirms mimicking of the pathological or physiologic condition.

[00302] Alternatively, when the at least one plated hepatic cell type comprises hepatocytes, responsiveness to glucagon, insulin, or a glucose substrate in the hepatocytes confirms mimicking of the physiologic condition. The glucose substrate can be, for example, glycerol, lactate, pyruvate, or combinations thereof (e.g., a combination of lactate and pyruvate).

Pathological Conditions

[00303] The pathological conditions of the liver that can be mimicked using the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 include, but are not limited to fatty liver disease, hepatitis C, hepatitis B, liver fibrosis, bacterial infection, viral infection, cirrhosis, and alcohol-induced liver disease.

[00304] When the pathological condition is fatty liver disease, the cell types can comprise hepatocytes, nonparenchymal hepatic cells, or combinations thereof. The nonparenchymal hepatic cells can include sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells, or combinations thereof.

[00305] When the pathological condition is fatty liver disease, the flow or hemodynamic pattern can be from a normal subject, a subject having fatty liver disease, or an animal genetically modified to model fatty liver disease.

[00306] Where the pathological condition is fatty liver disease and a porous membrane is used, hepatocytes can be plated on a first surface of the porous membrane. The porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane. The shear force is applied to the second surface of the porous membrane in the upper volume. Optionally, nonparenchymal hepatic cells can be plated on the second surface of the porous membrane, and the shear force is applied to the nonparenchymal hepatic cells in the upper volume. Optionally, an extracellular matrix component can be deposited on the first surface of the porous membrane, and subsequently hepatoctyes can be plated on the extracellular matrix component.

[00307] Where the pathological condition is fatty liver disease and a porous membrane is used, nonparenchymal hepatic cells can be plated on a second surface of a porous membrane. The porous membrane is suspended in the cell culture container such that a first surface of the porous membrane is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the first surface of the porous membrane and an upper volume comprising the nonparenchymal hepatic cells. The shear force is applied to the nonparenchymal hepatic cells in the upper volume. Optionally, an extracellular matrix component can be deposited on the first surface of the porous membrane, and subsequently hepatoctyes can be plated on the extracellular matrix component.

[00308] When the vascular pathological condition is fatty liver disease, the factor can comprise insulin, glucose, or a combination thereof. For example, the factor(s) can comprise insulin; glucose; or insulin and glucose.

[00309] When the pathological condition is diabetes, the cell type can comprise pancreatic β-cells, pancreatic α-cells, or a combination thereof; and the factor can comprise insulin, glucose, or insulin and glucose.

Physiologic Conditions

[00310] The physiologic conditions that can be mimicked using the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 include the physiologic conditions corresponding to any pathological

condition of interest. For example, a physiologic condition corresponding to fatty liver disease can be a healthy liver state, and a physiologic condition corresponding to atherosclerosis can be an atheroprotective state.

Flow Devices

[00311] The flow devices that can be used in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 are the same as described hereinabove in the section entitled "Flow Devices."

Hemodynamic Patterns

[00312] The hemodynamic patterns that can be used in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 can be derived from a subject or subjects having the pathological condition or a disease-promoting condition. The disease-promoting condition can comprise atrophy, calculi, choristoma, pathologic constriction, pathologic dilation, diverticulum, hypertrophy, polyps, prolapse, rupture, an arteriovenous fistula, or an appendage (e.g., a left atrial appendage).

[00313] The hemodynamic pattern can be derived from at least a portion of an artery, an arteriole, a vein, a venule, or an organ.

[00314] When a hemodynamic pattern is derived from at least a portion of an artery or an arteriole, the artery or arteriole can comprise a carotid artery, thoracic artery, abdominal artery, pulmonary artery, femoral artery, renal efferent artery, renal afferent artery, coronary artery, brachial artery, internal mammary artery, cerebral artery, aorta, pre-capillary arteriole, hepatic artery, anterior cerebral artery, middle cerebral artery, posterior cerebral artery, basilar artery, external carotid artery, internal carotid artery, vertebral artery, subclavian artery, aortic arch, axillary artery, internal thoracic artery, branchial artery, deep branchial artery, radial recurrent artery, superior epigastric artery, descending aorta, inferior epigastric artery, interosseous artery, radial artery, ulnar artery, palmar carpal arch, dorsal carpal arch, superficial or deep palmar arch, digital artery, descending branch of the femoral circumflex artery, descending genicular artery, superior genicular artery, inferior genicular artery, anterior tibial artery, posterior tibial artery, peroneal artery, deep plantar arch, arcuate artery, common carotid artery, intercostal arteries, left or right gastric artery, celiac trunk, splenic artery, common hepatic artery, superior mesenteric artery, renal artery, inferior

mesenteric artery, testicularis artery, common iliac artery, internal iliac artery, external iliac artery, femoral circumflex artery, perforating branch, deep femoral artery, popliteal artery, dorsal metatarsal artery, or dorsal digital artery.

[00315] When a hemodynamic pattern is derived from at least a portion of an vein or venule, the vein or venule can comprise a post-capillary venule, saphenous vein, hepatic portal vein, superior vena cava, inferior vena cava, coronary vein, Thesbian vein, superficial vein, perforator vein, systemic vein, pulmonary vein, jugular vein, sigmoid sinus, external jugular vein, internal jugular vein, inferior thyroid vein, subclavian vein, internal thoracic vein, axillary vein, cephalic vein, branchial vein, intercostal vein, basilic vein, median cubital vein, thoracoepigastric vein, ulnar vein, median antebranchial vein, inferior epigastric vein, deep palmar arch, superficial palmar arch, palmar digital vein, cardiac vein, inferior vena cava, hepatic vein, renal vein, abdominal vena cava, testicularis vein, common iliac vein, perforating branch, external iliac vein, internal iliac vein, external pudendal vein, deep femoral vein, great saphenous vein, femoral vein, accessory saphenous vein, superior genicular vein, popliteal vein, inferior genicular vein, great saphenous vein, small saphenous vein, anterior or posterior tibial vein, deep plantar vein, dorsal venous arch, or dorsal digital vein.

[00316] When a hemodynamic pattern is derived from at least a portion of an organ, the organ can comprise a liver, a kidney, a lung, a brain, a pancreas, a spleen, a large intestine, a small intestine, a heart, a skeletal muscle, an eye, a tongue, a reproductive organ, or an umbilical cord. The hemodynamic pattern is preferably derived from a liver.

[00317] The hemodynamic pattern can be derived from analysis of ultrasound data.

[00318] The hemodynamic pattern can be derived from analysis of magnetic resonance imaging (MRI) data.

[00319] The flow or the hemodynamic pattern can be time-variant.

[00320] The flow or the hemodynamic pattern can result from a physical change resulting from a pathological condition.

[00321] The flow or hemodynamic pattern can be derived from a subject wherein blood flow or a hemodynamic pattern has been altered as a direct or indirect effect of administration of a drug to a subject as compared to the flow or the hemodynamic pattern for the subject absent administration of the drug.

[00322] The flow or the hemodynamic pattern can be derived from an animal, such as a genetically modified animal or a human. Preferably, the pattern is derived from a human.

Cell Types

[00323] The cell types that can be used in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 include primary cells and immortalized cells. The primary cells or immortalized cells can comprise cells isolated from at least one subject having the pathological or physiologic condition, cells isolated from at least one subject having a risk factor for the pathological condition, cells isolated from at least one subject with a single nucleotide polymorphism linked to a pathological condition, cells isolated from at least one subject with an identified genotype linked to drug toxicity, or cells isolated from at least one subject with a single nucleotide polymorphism linked to drug toxicity.

[00324] The primary cells or the immortalized cells used in *in vitro* methods involving a physiologic condition comprise cells isolated from at least one subject having the physiologic condition, cells isolated from at least one subject having a risk factor for a pathological condition, cells isolated from at least one subject with a single nucleotide polymorphism linked to a pathological condition, cells isolated from at least one subject with an identified genotype linked to drug toxicity, or cells isolated from at least one subject with a single nucleotide polymorphism linked to drug toxicity.

[00325] The primary cells or immortalized cells used in *in vitro* methods involving a pathological condition can comprise cells isolated from at least one subject having the pathological condition, cells isolated from at least one subject having a risk factor for the pathological condition, cells isolated from at least one subject with a single nucleotide polymorphism linked to the pathological condition, cells isolated from at least one subject with an identified genotype linked to drug toxicity, or cells isolated from at least one subject with a single nucleotide polymorphism linked to drug toxicity.

[00326] The primary cells or immortalized cells used in *in vitro* methods involving a pathological condition can comprise cells isolated from at least one subject not having the pathological condition, cells isolated from at least one subject not having a risk factor for the pathological condition, cells isolated from at least one subject without a single nucleotide polymorphism linked to the pathological condition, cells isolated from at least one subject without an identified genotype linked to drug toxicity, or cells isolated from at least one subject without a single nucleotide polymorphism linked to drug toxicity.

[00327] The primary cells or immortalized cells used in *in vitro* methods involving a pathological condition can comprise cells isolated from at least one subject having a different pathological condition, cells isolated from at least one subject having a risk factor for a different pathological condition, or cells isolated from at least one subject with a single nucleotide polymorphism linked to a different pathological condition.

[00328] When the cells are isolated from at least one subject having a risk factor for the pathological condition, the risk factor can include, but is not limited to, smoking, age, gender, race, epigenetic imprinting, an identified genotype linked to the pathological condition, an identified single nucleotide polymorphism linked to the pathological condition, diabetes, hypertension, atherosclerosis, atherosclerotic plaque rupture, atherosclerotic plaque erosion, thoracic aortic aneurysm, cerebral aneurysm, abdominal aortic aneurysm, cerebral aneurysm, heart failure, stroke, Marfan syndrome, carotid intima-medial thickening, atrial fibrillation, kidney disease, pulmonary fibrosis, chronic obstructive pulmonary disease, pulmonary artery disease, pulmonary hypertension, hyperlipidemia, familial hypercholesterolemia, peripheral artery disease, arterial thrombosis, venous thrombosis (e.g., deep vein thrombosis), vascular restenosis, vascular calcification, myocardial infarction, obesity, hypertriglyceridemia, hypoalphalipoproteinemia, fatty liver disease, hepatitis C, hepatitis B, liver fibrosis, bacterial infection, viral infection, cirrhosis, liver fibrosis, or alcohol-induced liver disease.

[00329] The primary cells can include a cell lineage derived from stem cells (e.g., adult stem cells, embryonic stem cells, inducible pluripotent stem cells, or bone marrow-derived stem cells) or stem-like cells. The cell lineage derived from stem cells or stem-like cells can comprise endothelial cells, smooth muscle cells, cardiac myocytes, hepatocytes, neuronal cells, endocrine cells, pancreatic β-cells, pancreactic α-cells, or skeletal muscle cells.

[00330] The primary cells can comprise inducible pluripotent stem cell (iPSC)-derived cells from a subject having a pathological condition. For example, the iPSC-derived cells from a subject having a pathological condition can comprise iPSC-derived hepatocytes from a subject having familial hpercholesterolemia, glycogen storage disease type I, Wilson's disease, A1 anti-trypsin deficiency, Crigler-Najjar syndrome, progressive familial hereditary cholestasis, or hereditary tyrosinemia Type 1. Alternatively, the iPSC-derived cells from a subject having a pathological condition can comprise iPSC-derived vascular cells (e.g., iPSC-derived smooth muscle cells, iPSC-derived endothelial cells, or iPSC-derived endocardial

cells) from a subject having Hutchinson-Gilford progeria, Williams-Beuren syndrome, Fabry's disease, Susac's syndrome, systemic capillary leak syndrome, Gleich syndrome, intravascular papillary endothelial hyperplasia, sickle cell disease, or hepatic veno-occlusive disease.

[00331] Cell types for use in methods include vascular cells and hepatic cell.

[00332] Specific cell types for use in the methods include endothelial cells, hepatocytes, nonparenchymal hepatic cells, endothelial progenitor cells, stem cells, and circulating stem cells. The nonparenchymal hepatic cells include hepatic stellate cells, sinusoidal endothelial cells, and Kupffer cells. Preferably, the specific cell types can include endothelial cells, hepatocytes, sinusoidal endothelial cells, or a combination thereof.

[00333] The cell types for use in the methods can be animal cell types, such as cells from a genetically modified animal. The animal cell types are preferably human cell types. The human cell types can be selected on the basis of age, gender, race, epigenetics, disease, nationality, the presence or absence of one or more single nucleotide polymorphisms, a risk factor as described herein, or some other characteristic that is relevant to the pathological or physiologic condition.

[00334] The shear force applied in the methods can be applied indirectly to the at least one plated cell type.

[00335] The shear force applied in the methods can be applied directly to the at least one plated cell type.

[00336] The cell types, additional components such as extracellular matrix component, and the porous membrane are within the culture media (i.e., covered with culture media) in the methods.

[00337] The methods can further comprise analyzing at least one of the cell types for toxicity, inflammation, permeability, compatibility, cellular adhesion, cellular remodeling, cellular migration, or phenotypic modulation resulting from the drug or the compound.

Cell Culture Media

[00338] Standard cell culture media can be used in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939.

In Vivo *Factor Concentrations*

[00339] The physiologic *in vivo* concentrations of the factors for use in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 are well known in the art, as are the methods of determining these *in vivo* concentrations. Methods for determining *in vivo* concentrations of factors are available in the *United States Pharmacopeia* and in other literature.

[00340] A reported *in vivo* concentration range for a factor can vary depending upon the method used for determining the range, the source from which the factor is obtained (e.g., whole blood or serum), the medical condition of the patient (i.e., whether the patient has a pathological condition or physiologic condition), and time of day relative to normal sleep and eating schedule. However, it would be known to one of ordinary skill in the art that a concentration outside an *in vivo* physiological concentration range reported in the literature would be an *in vivo* pathological concentration using the method reported for determining the concentration. Likewise, a concentration below the lower endpoint or above the upper endpoint of an *in vivo* pathological concentration range reported in the literature would be an *in vivo* physiologic concentration using the method reported for determining the concentration; whether the *in vivo* physiologic concentration is below the lower endpoint or above the upper endpoint will depend upon the factor.

Extracellular Matrix Components

[00341] Extracellular matrix components for use in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 can comprise heparan sulfate, chondroitin sulfate, keratan sulfate, hyaluronic acid, a collagen, an elastin, a fibronectin, a laminin, a vitronectin, or combinations thereof. Collagen is a preferred extracellular matrix component, and is preferably the type of collagen that is present in the *in vivo* environment of the cell type or cell type(s) that are plated for a particular pathological or physiologic condition.

[00342] The extracellular matrix component can be secreted by fibroblasts, chondrocytes, or osteoblasts plated on the surface within the cell culture container.

Drugs or Compounds

[00343] The drug or compound for use in the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 involving testing of a drug or compound can comprise any drug or compound.

[00344] The concentration of the drug or the compound in the culture media is suitably within the concentration range of the drug or the compound that achieves the effect *in vivo*. For example, the concentration of the drug or the compound in the culture media is suitably within the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound.

Sera

[00345] In any of the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 that involve adding a factor to the culture media or adding a drug or compound to the culture media, the step of adding the factor to the culture media or the step of adding the drug or a compound to the culture media can comprise adding sera from a subject to the culture media, wherein the sera comprises the factor, the drug, or the compound.

[00346] The subject can be an animal, e.g., as a genetically modified animal or a human. Preferably, the sera is derived from a human subject.

[00347] The sera can be from a subject having a physiologic condition or a subject having a pathological condition. For example, where the sera is from a subject that has a pathological condition, the pathological condition can comprise advanced inflammation, atherosclerosis, diabetic nephropathy, diabetic neuropathy, diabetic retinopathy, hypertension, hypertensive encephalopathy, hypertensive retinopathy, fatty liver disease, hypertension, heart failure, stroke, Marfan syndrome, carotid intima-medial thickening, atrial fibrillation, kidney disease, pulmonary fibrosis, chronic obstructive pulmonary disease, hyperlipidemia, hypercholesterolemia, diabetes, atherosclerotic plaque rupture, atherosclerotic plaque erosion, thoracic aortic aneurysm, cerebral aneurysm, abdominal aortic aneurysm, cerebral aneurysm, pulmonary artery disease, pulmonary hypertension, peripheral artery disease, arterial thrombosis, venous thrombosis (e.g., deep vein thrombosis), vascular restenosis, vascular calcification, myocardial infarction, obesity, hypertriglyceridemia, hypoalphalipoproteinemia, hepatitis C, hepatitis B, liver fibrosis, bacterial infection, viral infection, cirrhosis, liver fibrosis, or alcohol-induced liver disease.

Effect on the Physiologic or Pathological Condition

[00348] In methods of testing a drug or a compound for an effect described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939, the effect can comprise an effect on a physiologic condition or an effect on a pathological condition. For example, the effect on the physiologic condition or the pathological condition can be a toxic effect, a protective effect, a pathologic effect, a disease-promoting effect, an inflammatory effect, an oxidative effect, an endoplasmic reticulum stress effect, a mitochondrial stress effect, an apoptotic effect, a necrotic effect, a remodeling effect, a proliferative effect, an effect on the activity of a protein, such as inhibition of a protein or activation of a protein, or an effect on the expression of a gene, such as an increase in the expression of the gene or a decrease in the expression of the gene.

Multiple Cell Type Configurations for the Flow Device

[00349] The methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 can further comprise perfusing culture media, factors, drugs or compounds into and out of the cell container.

[00350] When the surface within the cell culture container comprises a porous membrane suspended in the cell culture container, the method can further include the step of plating at least one cell type on a surface within the cell culture container comprising plating a first cell type on a first surface of a porous membrane, and optionally plating a second cell type on a second surface of the porous membrane, wherein the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the first cell type and an upper volume comprising the optional second cell type. The porous membrane can be adapted to permit fluid communication of the cell culture media and physical interaction and communication between cells of the first cell type and cells of the optional second cell type. The shear force is applied to the second cell type or the second surface of the porous membrane in the upper volume. The method can further comprise perfusing culture media into and out of the upper volume and perfusing culture media into and out of the lower volume. The method can further comprise perfusing a drug or the compound into at least one of the upper volume and the lower volume.

[00351] When the surface within the cell culture container comprises a porous membrane suspended in the cell culture container, the method can further include the step of plating at least one cell type on a surface within the cell culture container comprising optionally plating a first cell type on a first surface of a porous membrane, and plating a second cell type on a second surface of the porous membrane, wherein the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the optional first cell type and an upper volume comprising the second cell type. The porous membrane can be adapted to permit fluid communication of the cell culture media and physical interaction and communication between cells of the optional first cell type and cells of the second cell type. The shear force is applied to the second cell type in the upper volume. The method can further comprise perfusing culture media into and out of the upper volume and perfusing culture media into and out of the lower volume. The method can further comprise perfusing a drug or the compound into at least one of the upper volume and the lower volume.

[00352] The inlets and outlets in the cell culture container can be within the portions of the cell culture container defining the upper and lower volumes.

[00353] The methods described in this section can further comprise analyzing at least one of the first cell type or the second cell type for toxicity, inflammation, permeability, compatibility, cellular adhesion, cellular remodeling, cellular migration, or phenotypic modulation resulting from the drug or the compound.

[00354] These methods can further comprise plating a third cell type on a surface of the container or the first surface or second surface of the porous membrane, suspending a third cell type in the culture media within the upper volume, or suspending a third cell type in the culture media within the lower volume.

[00355] These methods can further comprise plating a fourth cell type on a surface of the container or the first or second surface of the porous membrane, suspending a fourth cell type in the culture media within the upper volume, or suspending a fourth cell type in the culture media within the lower volume.

[00356] These methods can further comprise plating a fifth cell type on a surface of the container or the first or second surface of the porous membrane, suspending a fifth cell type in the culture media within the upper volume, or suspending a fifth cell type in the culture media within the lower volume.

[00357] The first, second, third, fourth and fifth cell types can be various primary or immortalized cell types as described in the section above regarding cell types.

[00358] In each of these combinations, the cells of the third cell type, the cells of the fourth cell type or the cells of the fifth cell type can be adhered to the bottom surface of the container.

Definitions

[00359] With respect to the methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939, the term "factor" means a biological substance that contributes to the production of a pathological or physiologic condition. Preferably, the factor provides a change in a level of a marker of the pathological or physiologic condition in the at least one plated cell type or in the culture media upon application of the shear force, as compared to the level of the marker in the at least one plated cell type or in the culture media in the absence of application of the shear force.

[00360] The term "pathological condition" means an abnormal anatomical or physiological condition, which includes the objective or subjective manifestation of a disease.

[00361] The term "physiologic condition" means a normal medical state that is not pathologic, and can be a medical state characteristic of or conforming to the normal functioning or state of the body or a tissue or organ.

Physiologic Liver Model

[00362] The methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 can be used to create a physiologic *in vitro* model of the liver. In such methods, hepatocytes are plated on a surface within a cell culture container, and shear forces are applied indirectly to the plated hepatocytes. For example, the hepatocytes are suitably plated on a first surface of a porous membrane, where the porous membrane is suspended in a cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane. The shear force is applied to the second surface of the porous membrane in the upper volume of the container.

Thus, the configuration of cells in the device is based on *in vivo* microarchitecture of hepatic lobules.

[00363] In hepatic lobules *in vivo*, cords of hepatocytes are separated from sinusoidal blood flow by a filtering layer of sinusoidal endothelial cells and a layer of extracellular matrix. The layer of extracellular matrix provides for anchorage of the hepatocytes, is involved in signaling, and provides a reservoir of cytokines and growth factors. The hepatocytes have a polarized morphology and biliary canaliculi are present in the hepatocyte layer. Sinusoidal blood flow and interstitial blood flow provide for oxygen and nutrient transport.

[00364] Figure 11 depicts an exemplary configuration used in the *in vitro* liver model and is described above. The porous membrane acts analogously to the filtering layer of sinusoidal endothelial cells which is present in the liver. The hepatocytes are shielded from direct effects of flow, as they would be *in vivo*. Inlets and outlets in the upper and lower volumes within the cell culture container allow for the continuous perfusion of culture media and for perfusion of drugs or compounds into and out of the cell culture media. Application of the shear force creates controlled hemodynamics that regulate interstitial flow and solute transfer through the porous membrane. In the *in vitro* models described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939, the hepatocytes maintain their polarized morphology and bile canaliculi.

[00365] At least one layer of one or more extracellular matrix components (e.g., a collagen gel) can suitably be deposited on a first surface of the porous membrane. The hepatocytes are then plated on the extracellular matrix component(s). One or more additional layers of the extracellular matrix component(s) can then be deposited on top of the hepatocytes, such that the hepatocytes are substantially surrounded by the extracellular matrix component(s). The extracellular matrix component suitably comprises heparan sulfate, chondroitin sulfate, keratan sulfate, hyaluronic acid, a collagen, an elastin, a fibronectin, a laminin, a vitronectin, or combinations thereof. For example, the extracellular matrix component can comprise collagen.

[00366] One or more additional cell types can be plated on a surface within the cell culture container or suspended in the culture media. For example, nonparenchymal hepatic cells are suitably plated on the second surface of the porous membrane, and the shear force is applied to the plated non-parenchymal cells. The nonparenchymal cells may include hepatic stellate cells, sinusoidal endothelial cells, Kupffer cells, or combinations thereof. The

hepatocytes and nonparenchymal hepatic cells are suitably primary cells isolated from the liver of an animal, for example from the liver of a human. Alternatively, the hepatocytes and/or the nonparenchymal hepatic cells are immortalized cells.

[00367] Media is suitably continuously perfused on both sides of the porous membrane, while shear forces, derived from a range of physiological blood flow values, are continuously applied to the second surface of the porous membrane or to the plated nonparenchymal hepatic cells. The shear forces applied to the second surface of the porous membrane mimic the flow through hepatic sinusoids which occurs *in vivo*. The shear rate is suitably about 0.1 dynes/cm^2 to about 3.0 dynes/cm^2, about 0.2 dynes/cm^2 to about 2.5 dynes/cm^2, about 0.3 dynes/cm^2 to about 1.0 dynes/cm^2 or about 0.4 dynes/cm^2 to about 0.8 dynes/cm^2. For example, the shear rate can be about 0.6 dynes/cm^2. Alternatively, the shear rate can be about 2.0 dynes/cm^2.

[00368] In the physiologic *in vitro* liver model, one or more factors are present in the culture media. These one or more factors can be added to the media at concentrations which are capable of maintaining the mimicking of the physiologic liver condition *in vitro* for a period of time under the shear force, where the same concentrations of these factors are incapable of maintaining the mimicking of the physiologic liver condition *in vitro* for the period of time in the absence of the shear force. For example, the factors may comprise insulin, glucose, or a combination of insulin and glucose. The glucose and insulin are suitably present in reduced concentrations as compared to the concentrations which are typically used in static cultures (about 17.5 mM glucose and about 2 μM insulin). For example, the glucose may be present in the culture media at a concentration of about 5 mM to about 10 mM, or at a concentration of about 5.5 to about 7 mM, e.g., at a concentration of about 5.5 mM. The insulin may be present in the culture media at a concentration of about 0.05 nM to about 5 nM, for example about 0.1 nM to about 3 nM, or about 0.5 to about 2.5 nM, e.g., at a concentration of about 2 nM. The one or more factors are suitably added to the culture media before or concurrently with application of the shear force.

[00369] The concentrations of the one or more factors are suitably capable of maintaining the mimicking of the physiologic liver condition *in vitro* for at least about 7 days, at least about 14, days, at least about 21 days, at least about 30 days, or longer.

[00370] Mimicking of the physiologic liver condition can be assessed by a number of methods. In general, a change in a level of a marker of the physiologic liver condition in the hepatocytes or nonparenchymal hepatic cells or in the culture media upon application of

the shear force, as compared to the level of the marker in the hepatocytes or nonparenchymal hepatic cells or in the culture media in the absence of application of the shear force confirms mimicking of the physiologic liver condition. For example, mimicking of the physiologic liver condition can be assessed by examining the hepatocytes or nonparenchymal hepatic cells for the expression of genes or proteins involved in maintaining the liver in a physiologic state (e.g., in hepatocytes, metabolic and insulin/glucose/lipid pathway genes); examining the hepatocytes for lipid accumulation; examining the hepatocytes or nonparenchymal hepatic cells for changes in differentiated function (e.g., in hepatocytes, measuring urea and albumin secretion); examining the hepatocytes or nonparenchymal hepatic cells for changes in metabolic activity (e.g., in hepatocytes, using cytochrome p450 assays) or transporter activity; or by examining the hepatocytes or nonparenchymal hepatic cells for morphological changes. The physiologic condition of the liver can also be assessed by comparing the response of the hepatocytes or nonparenchymal hepatic cells to xenobiotics, nutrients, growth factors or cytokines to the *in vivo* liver response to the same xenobiotics, nutrients, growth factors or cytokines.

[00371] As described further in Example 12 below, unlike hepatocytes cultured under static conditions, hepatocytes cultured in the physiologic *in vitro* liver model described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 maintain their responsiveness to glucagon, insulin, and glucose substrates. Thus, responsiveness to glucagon, insulin, or one or more glucose substrates (e.g., using a gluconeogenesis assay) can also be used to assess mimicking of the physiologic liver condition. Suitable glucose substrates include glycerol, lactate, pyruvate, or combinations thereof (e.g., a combination of lactate and pyruvate). Moreover, because the hepatocytes maintain responsiveness to glucagon, the physiologic *in vitro* liver model can be used for *in vitro* testing of drugs that interact with the glucagon receptor (e.g., glucagon receptor antagonists).

[00372] In addition, hepatocytes cultured in the physiologic *in vitro* liver model described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 display induction and toxicity responses to drugs at concentrations much closer to *in vivo* and clinical C_{max} levels than static culture systems. Thus, this model can be used for *in vitro* testing of drugs and compounds at concentrations within the concentration range of the drug or compound that achieves an effect *in vivo*.

Fatty Liver

[00373] The methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 can also be used to create an *in vitro* model of fatty liver disease. Lipid regulation within hepatocytes is a complex and dynamic process. Triglyceride buildup can occur as a consequence of increased fatty acid uptake from a high fat diet, increased peripheral lipolysis, or from increased *de novo* lipogenesis. Insulin and glucose are key regulators of *de novo* lipogenesis and contribute to increased triglyceride content within hepatocytes by stimulating triglyceride synthesis as well as inhibiting fatty acid metabolism by beta oxidation.

[00374] Non-alcoholic fatty liver disease (NAFLD) is correlated with obesity, type II diabetes, and metabolic syndrome in the presence of insulin resistance. NAFLD is characterized by hepatic steatosis (excessive lipid accumulation in the liver) that if left untreated progresses to inflammatory changes (steatohepatitis) and cirrhosis. Many animal models induce steatosis through a hyperglycemic-hyperinsulinemic environment (e.g., through use of a low fat/high carbohydrate diet to stimulate lipogenesis). However, current *in vitro* hepatocyte models lack an adequate insulin-glucose response to induce the same, probably on account of the superphysiological levels of insulin/glucose required to maintain hepatocytes in culture under static conditions. Such *in vitro* models fail to induce fatty changes in hepatocytes through insulin and glucose, perhaps due to impaired insulin responsiveness of hepatocytes under static culture conditions and rapid dedifferentiation of the hepatocytes *in vitro*.

[00375] By contrast, as described above with respect to the physiological liver model, hepatocytes cultured in the presence of controlled liver-derived hemodynamics and transport retain differentiated function, morphology, and response at physiological glucose and insulin levels. In this system, introducing high concentrations of insulin and glucose (a "disease milieu") induces fatty changes in the hepatocytes. Thus, controlled hemodynamics and transport produces a more physiological response to insulin and glucose in the hepatocytes, thereby inducing the fatty changes associated with steatosis in a hyperinsulemic, hyperglycemic environment as is typically seen initially under insulin resistant conditions of diabetes. In addition, hepatocytes cultured in the presence of controlled hemodynamics and transport display induction and toxicity responses to drugs at concentrations much closer to *in vivo* and clinical C_{max} levels than static culture systems. This system therefore provides an *in vitro* model of fatty liver disease.

[00376] In this model, the hepatocytes are generally plated in the same manner as described above for the physiological liver model. Hepatocytes are plated on a surface within a cell culture container, and shear forces are applied indirectly to the plated hepatocytes. For example, the hepatocytes are suitably plated on a first surface of a porous membrane, where the porous membrane is suspended in a cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane. The shear force is applied to the second surface of the porous membrane in the upper volume of the container.

[00377] At least one layer of one or more extracellular matrix components can suitably be deposited on the first surface of the porous membrane. The hepatocytes are then plated on the extracellular matrix component(s). One or more additional layers of the extracellular matrix component(s) can then be deposited on top of the hepatocytes, such that the hepatocytes are substantially surrounded by the extracellular matrix component(s). The extracellular matrix component suitably comprises heparan sulfate, chondroitin sulfate, keratan sulfate, hyaluronic acid, a collagen, an elastin, a fibronectin, a laminin, a vitronectin, or combinations thereof. For example, the extracellular matrix component can comprise collagen.

[00378] One or more additional cell types can be plated on a surface within the cell culture container or suspended in the culture media. For example, nonparenchymal hepatic cells are suitably plated on the second surface of the porous membrane, and the shear force is applied to the plated non-parenchymal cells. The nonparenchymal cells may include hepatic stellate cells, sinusoidal endothelial cells, Kupffer cells, or combinations thereof. The hepatocytes and nonparenchymal hepatic cells are suitably primary cells isolated from the liver of an animal, for example from the liver of a human. Alternatively, the hepatocytes and/or the nonparenchymal hepatic cells are immortalized cells.

[00379] Media is suitably continuously perfused on both sides of the porous membrane, while shear forces, derived from a range of physiological blood flow values, are continuously applied to the second surface of the porous membrane or to the plated nonparenchymal hepatic cells. The shear forces applied to the second surface of the porous membrane mimic the flow through hepatic sinusoids which occurs *in vivo*. The shear rate is suitably about 0.1 dynes/cm^2 to about 3.0 dynes/cm^2, about 0.2 dynes/cm^2 to about 2.5 dynes/cm^2, about 0.3 dynes/cm^2 to about 1.0 dynes/cm^2 or about 0.4 dynes/cm^2 to about 0.8

dynes/cm^2. For example, the shear rate can be about 0.6 dynes/cm^2. Alternatively, the shear rate can be about 2.0 dynes/cm^2.

[00380] In the *in vitro* fatty liver model, one or more factors are present in the culture media. These one or more factors are added to the media at concentrations which are capable of maintaining the mimicking of fatty liver disease *in vitro* for a period of time under the shear force, the same concentration of factor being incapable of maintaining the mimicking of fatty liver disease for the period of time in the absence of the shear force. The factors may comprise, for example, insulin, glucose, or a combination thereof. The glucose is suitably present in the culture media at a concentration of about 10 mM to about 25 mM, about 12 mM to about 20 mM, or about 14 mM to about 18 mM, e.g., about 17.5 mM. The insulin is suitably present in the culture medium at a concentration of about 1 μM to about 3 μM, about 1.5 μM to about 2.5 μM, or about 1.8 μM to about 2.2 μM, e.g., about 2 μM. The one or more factors are suitably added to the culture media before or concurrently with application of the shear force.

[00381] The concentrations of the one or more factors are suitably capable of maintaining the mimicking of fatty liver disease condition *in vitro* for at least about 7 days, at least about 14, days, at least about 21 days, at least about 30 days, or longer.

[00382] Mimicking of fatty liver disease can be assessed by a number of methods. In general, a change in a level of a marker of fatty liver disease in the hepatocytes or nonparenchymal hepatic cells or in the culture media upon application of the shear force, as compared to the level of the marker in the hepatocytes or nonparenchymal hepatic cells or in the culture media in the absence of application of the shear force confirms mimicking of fatty liver disease. For example, mimicking of fatty liver disease can be assessed by examining the hepatocytes or nonparenchymal hepatic cells for the expression of genes or proteins involved in the fatty liver disease state (e.g., in hepatocytes, metabolic and insulin/glucose/lipid pathway genes); examining the hepatocytes for lipid accumulation (e.g., in hepatocytes, measuring triglyceride levels or visualizing lipid droplets); examining the hepatocytes or nonparenchymal hepatic cells for changes in differentiated function (e.g., in hepatocytes, measuring urea and albumin secretion); examining the hepatocytes or nonparenchymal hepatic cells for changes in metabolic activity (e.g., in hepatocytes, using cytochrome p450 assays) or transporter activity; or by examining the hepatocytes or nonparenchymal hepatic cells for morphological changes. Sequelae to fatty liver changes can also be assessed by

measuring the changes in oxidative state of the hepatocytes and the changes in surrounding extracellular matrix composition and amount.

[00383] The methods described in U.S. Patent Application Publication No. 2013/0309677 and PCT Publication No. 2013/0158939 are further illustrated by Examples 12–14 below.

DEFINITIONS

[00384] For purposes of the inventions described herein, the term "hemodynamic" means blood flow that mimics the blood flow *in vivo* in a tumor or tissue of interest. For example, when blood flow in the microvasculature of a tumor, the acceleration/deceleration rates, flow reversal, forward basal flow, etc. are some parameters characterizing arterial hemodynamic flow. In some tissues, such as the liver, a constant blood flow may be used to characterize *in vivo* hemodynamics.

[00385] The term "subject" means an animal (e.g., a genetically modified animal or a human). The animal can include a mouse, rat, rabbit, cat, dog, primate, guinea pig, hamster, monkey, cow, pig, horse, goat, sheep, bird or fish, or any animal typically used in medical research.

[00386] Having described the invention in detail, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims.

EXAMPLES

[00387] The following non-limiting examples are provided to further illustrate the present invention.

Example 1: Plating and Culturing Methods

[00388] Porous membranes used for plating were porous membranes of TRANSWELL cell culture inserts (polycarbonate, 10 μm or 18 μm thickness and 0.4 μm pore diameter, 75 mm insert diameter, custom ordered from Corning). To prepare the inserts for cell plating, both surfaces of the porous membrane were coated with gelatin (0.14% solution in sterile water). The porous membranes separate the cell types in the cultures but allow for cell-cell interactions to occur through the pores of the membrane.

[00389] The following cell types were used: human fibroblasts (Hs888Lu lung fibroblasts, American Type Culture Collection (ATCC) CCL-211), dtumor cells (A549 human non-small cell carcinoma (NSCLC) tumor cells, ATCC CL-185), and human dermal microvascular endothelial cells (HMVECad, Gibco, catalogue # C-011-5C). Each of these cell lines was maintained, passaged and plated in DMEM base (without phenol red) containing 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin (P), 100 μg/mL streptomycin (S), 2 mM L-glutamine (L-glut), 1 mM sodium pyruvate (NaP), 1X non-essential amino acids (NEAA, HyClone SH30238.011), and 4.5 g/L D-glucose.

[00390] The human fibroblasts were plated on the lower surface of the porous membrane of a cell culture insert at a plating density of 3.0 X 10^3 cells/cm^2 and allowed to adhere for at least two hours in a humidified chamber at 37°C with 5% CO_2.

[00391] To generate tri-cultures, a secondary porous membrane was cut from a 75 mm insert (TRANSWELL, polycarbonate, 10 μm or 18 μm thickness and 0.4 μm pore diameter, custom ordered from Corning) and prewet with a collagen solution (2.0 mg/mL rat tail collagen in 1X PBS, pH 7.4) by dipping the membrane into a 100 mm dish containing 1.0 mL of collagen solution, wetting both sides of the membrane and allowing any excess collagen solution to drip off the membrane. The secondary membrane was placed on top of the fibroblasts plated on the lower surface of the porous membrane of the cell culture insert and allowed to adhere and the collagen to solidify for one hour in a humidified chamber at 37°C with 5% CO_2. The secondary membrane separating the stromal fibroblasts and the tumor cells was needed to perform the RNAseq transcriptomics described hereinbelow, but can be omitted as shown, for example, in the configurations depicted in Figures 5–8.

[00392] Three plating conditions were used: (1) a plating configuration that was substantially free of exogenously added ECM (referred to in the Examples and Figures herein as "no collagen," "NC," "no ECM," or "no matrix"); (2) a plating configuration wherein the tumor cells were plated on a layer of exogenously added collagen (referred to in the Examples and Figures herein as "collagen layer" or "CL"); and (3) a plating configuration wherein the tumor cells were plated on a layer of exogenously added collagen, and wherein an additional layer of collagen was then added on top of the plated tumor cells such that the collagen substantially surrounded the tumor cells (referred to in the Examples and Figures herein as a "collagen sandwich" or CS).

[00393] In experiments where the plating configuration was substantially free of exogenous ECM, tumor cells were plated directly onto the opposing surface secondary

membrane (i.e., the surface not in contact with the fibroblasts). In this configuration, the only exogenous ECM added to the cell culture container was the collagen into which the secondary membrane was immersed.

[00394] In experiments where a collagen layer or collagen sandwich configuration was desired, 900 μL of collagen solution (2.0 mg/mL rat tail collagen in 1X PBS, pH 7.4) was applied evenly to the prewet opposing surface of the secondary membrane (i.e., the surface not in contact with the fibroblasts) and allowed to adhere and collagen to solidify for one hour in a humidified chamber at 37°C with 5% CO_2. This generated an approximately 200 micron thick collagen layer. This concentration of collagen produces a gel with a rigidity that mimics the stiffness of the tumor microenvironment.

[00395] Human tumor cells were then plated on the collagen layer on the surface of the secondary membrane at a plating density 3.0 x 10^3 cells/cm^2 and allowed to adhere for at least two hours in a humidified chamber at 37°C with 5% CO_2. After cells had adhered, the cell culture inserts were inverted and placed into a 100 mm culture dish containing 15 mL DMEM base (without phenol red) +10% FBS, P/S, L-glut, NaP, NEAA and D-glucose (9 mL of the culture medium was in the lower volume, and 6 mL of the culture medium was in the lower volume).

[00396] If a collagen sandwich was desired (i.e., a plating configuration wherein collagen substantially surrounds the cells plated on the lower surface of the porous membrane of the cell culture insert), an additional 900 μL of collagen solution (2.0 mg/mL rat tail collagen in 1X PBS, pH 7.4) was evenly applied on top of tumor cells and allowed to solidify for one hour in a humidified chamber at 37°C with 5% CO_2. This generated an approximately 200 micron thick layer of collagen on top of the cells plated on the lower surface of the porous membrane of the cell culture insert). After this top layer of collagen had solidified, the cell culture inserts were inverted and placed into a 100 mm culture dish containing 15 mL DMEM base (without phenol red) +10% FBS, P/S, L-glut, NaP, NEAA and D-glucose (9 mL of the culture medium was in the lower volume, and 6 mL of the culture medium was in the lower volume).

[00397] In all configurations, following plating of the fibroblasts and tumor cells, cells were allowed to grow for 48 hours in a humidified chamber at 37°C with 5% CO_2. Human endothelial cells were then plated on the upper surface of the porous membrane of the cell culture insert at a plating density of 5.0 X 10^4 cells/cm^2 and allowed to adhere for 24

hours in a humidified chamber at 37°C with 5% CO_2 under static conditions The resulting tri-culture plating configuration is illustrated in FIG. 9.

[00398] After endothelial cells had adhered, the tri-cultures were prepared for experimental hemodynamics and transport. Tri-cultures of tumor cells, fibroblasts, and endothelial cells were either maintained under static conditions (for static controls) or placed into a cone-and-plate device, the cone was lowered into the upper volume, and the cone was rotated to apply a shear force upon the endothelial cells. In cultures subjected to shear, transport was controlled in the system by perfusing cell culture medium into and out of both the upper and lower volumes of the cell culture dish via inlets 17 and outlets 19 in the upper and lower volumes, as depicted in FIG. 9. In experiments wherein drugs were used, drug solutions were added to the upper volume, which represents the vascular compartment.

[00399] The correlation of color Doppler ultrasound with histologic specimens from both benign and malignant tumors suggests that constant flow is more representative of the true neovascularization of malignant lung cancers (Hsu et al., 2007; Hsu et al. 1996; Görg et al. 2003). A monophasic low-impedance waveform more characteristic of peripheral bronchial artery blood flow was selected for application to the tri-cultures. Blood flow in this region is slower and lacks a significant systolic/diastolic variation, as illustrated in FIG. 4. For contrast, the shear stress pattern from a pulmonary lesion near the pulmonary artery is illustrated in FIG. 4.

Example 2: Morphology of Cells Cultured in In Vitro *Tumor Microenvironment Tri-Cultures*

[00400] Tri-cultures prepared as described above and as illustrated in FIG. 9 were fixed after seven days of hemodynamic shear stress in 4% Paraformaldehyde (Electron Microscopy Sciences) for 20 minutes at room temperature (RT), washed in phosphate Buffer saline (PBS) with Calcium and Magnesium (Fisher Scientific) and stored at 4°C until processed. Samples were permeabilized with 0.1% Triton for 20 min, and stained with ALEXA FLUOR 488 (a fluorescent dye)-labeled Phalloidin (1:100; Life Technologies) to stain for F-actin and TO-PRO-3 nuclear stain (1:2000; Life Technologies) for one hour at room temperature. After three washes with PBS, samples were mounted between coverslips using FLUOROMOUNT G (an aqueous mounting medium, Southern Biotech). Images were taken with a Nikon ECLIPSE Ti Confocal Microscope using 20X oil immersion objectives.

[00401] Confocal microscopy images are provided in Figure 13. Figures 13A, 13B, and 13C the morphology of the human dermal microvascular endothelial cells (FIG. 13A),

the human fibroblasts (FIG. 13B) and the human A549 NSCLC tumor cells (FIG. 13C) plated in the collagen sandwich plating condition. Figures 13D, 13D, and 13F show the morphology of the A549 tumor cells under the three different plating conditions: no ECM (FIG. 13D), collagen layer (FIG. 13E), and collagen sandwich (FIG. 13F). The phenotype of the A549 cells in the collagen sandwich was spheroidal whereas in the no ECM condition, the A549 cells pile up on one another but do not form spheres.

Example 3: Tumor Cell Growth in In Vitro *Tumor Microenvironment Tri-Cultures*

[00402] The growth rate of the human lung carcinoma cell line A549 (ATCC, Manassas, VA) was determined in multiple matrix conditions when exposed to tumor capillary hemodynamics. Cells were plated as shown in FIG. 9. Hs888Lu human lung fibroblasts were plated onto the porous membrane of the cell culture insert at an initial plating density of 3×10^3 cells/cm^2. A secondary membrane was applied as described above in Example 1 and A549 tumor cells were then seeded at an initial density of 3×10^3 cells/cm^2 in each of the three matrix conditions as described above in Example 1: (1) cells plated directly onto the secondary membrane (no matrix); (2) cells plated onto single layer of collagen (collagen layer); or (3) cells plated in a collagen sandwich. After 48 hours of incubation in a static environment, dermal microvascular endothelial cells were plated on the upper surface of the porous membrane of the cell culture insert at an initial plating density of 5×10^4 cells/cm^2. Cultures were cultured for up to seven days under static conditions or subjected to hemodynamic flow and transport. For static cultures, cell number was determined at the time of seeding (Day 0) and days 2, 4 and 7 using the CYQUANT Cell Proliferation Assay Kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR). For cultures subjected to hemodynamic flow and transport, cell number was determined on the day cultures were subjected to hemodynamic flow and transport (Day 0) and on days 2, 4, and 7 of hemodynamic flow and transport. Data are shown in FIGs. 14A and 14C and are presented as the mean number of cells from duplicate cultures for each of the three matrix conditions. For each condition, the growth rates were compared to the growth rate of A549 cells grown in plastic two-dimensional (2D) tissue culture dishes without matrix and in the absence of hemodynamic flow and transport.

[00403] As shown in Figure 14A, hemodynamic flow attenuated the growth rate of A549 tumor cells grown in the tri-cultures in collagen sandwiches as compared to static 2D culture. There has been a long standing disconnect between the doubling time of cells grown

in static 2D cultures and the doubling time of *in vivo* tumors (Cifone, 1982), be that in xenograft tumors derived from cell lines or the rate of tumor growth in patients. When the growth rate of A549 NSCLC cells grown in static 2D cultures and the tri-cultures subjected to hemodynamic conditions were compared, the tumor cell growth in the tri-cultures subjected to hemodynamic conditions was diminished relative to growth in the static 2D system (Figure 14A). The growth rate of A549 cells in static 2D conditions was 8-fold higher in phase II than in the tri-cultures subjected to hemodynamic conditions. While the growth rate of the A549 cells in the tri-cultures subjected to hemodynamic conditions still exceeds the rate of A549 xenografts (Basu et al., 2011; Chou et al., 2008; Li et al. 2013), the growth rate in the tri-cultures subjected to hemodynamic conditions was closer to the physiologic rate than that of static 2D cultures. 14B. FIG 14B shows the qualitative differences in the growth rate between A549 cells grown in static 2-dimensional cultures ("static"), the tri-cultures subjected to hemodynamic conditions ("*In vitro* tumor microenvironment"), and xenografts. The darkly shaded spheres in FIG. 14B represent the starting tumor size, and the lightly shaded areas and arrows represent tumor growth over time.

[00404] FIG. 14C illustrates that the decrease in growth occurred in all three matrix conditions examined. Thus, hemodynamic flow and transport, not extracellular matrix, appear to be the driving contributors effecting growth rate.

Example 4: Assessment of Cell Density and Monolayer Integrity of Endothelial Cells

[00405] Endothelial cell density and monolayer integrity of endothelial cells cultured according to the methods described above in Example 1 can be evaluated by fixing and immunostaining the endothelial cells on the porous membrane for the endothelial junction protein VE-cadherin and examining the monolayer by confocal microscopy.

Example 5: Morphological Assessment of Tumor Cells

[00406] To assess the morphology of tumor cells cultured using the methods described above in Example 1, morphologies of the tumor cells can be determined by immunostaining the tumor cells on the porous membrane for E-cadherin and for actin using fluorescently labeled phalloidin. To quantitate the extension of invasive structures (invadopodia) through the pores of the porous membrane, the cultures can be fixed and immunostained for E-cadherin (for the tumor cells) and VE-cadherin (for endothelial cells), and a cross-section of the porous membrane can be analyzed by confocal microscopy.

Example 6: Endothelial Cell Monolayer Permeability

[00407] In *in vivo* tumor-vasculature regions, an increase in vessel permeability occurs due to the secretion of numerous growth factors from the tumor cells (Mukaida et al., 2012; Bradford et al., 2013). To demonstrate that tumor cells alter endothelial cell barrier function in the tri-culture systems described above in Examples 1, permeability assays were performed to measure increases in endothelial monolayer permeability. In this assay, horseradish peroxidase (HRP) was added to the upper volume and accumulation of HRP in the presence and absence of tumor cells was measured over time.

[00408] After establishing tumor cell/fibroblast/endothelial cell cocultures as described in Example 1 and as illustrated in FIG. 9, a known mass of low molecular weight HRP was added to the upper volume and allowed to diffuse. Following a 15–60 minute incubation period, media samples were removed from the lower volume. Application of shear stress upon the endothelial cells was continued during this process. HRP that diffused through the endothelial cells, porous membranes, fibroblasts, and A549 tumor cells was detected by guaiacol oxidation. Endothelial cell monocultures (grown on porous membranes without the tumor cells) that were subjected to the shear stress were assayed in parallel as a control.

[00409] The results of this assay are provided in Figure 15 and demonstrate that the NSCLC A549 cells increase endothelial cell permeability. Relative HRP accumulation in the absence and presence of NSCLC A549 cells is shown. In Figure 15, "no tumor cells" indicates the endothelial cell monocultures, and "tumor cells" indicates the tumor cell/fibroblast/endothelial cell cocultures.

Example 7: Transcriptosomic Profile of Tumor Cells in In Vitro *Tumor Microenvironment Tri-Cultures*

[00410] Next-generation RNA sequencing (RNA-seq) was used to compare the transcriptosome of A549 tumor cells grown under three different conditions: (1) in static two dimensional cultures; (2) in xenografts in propagated subcutaneously in athymic nude mice; and (3) in the tri-cultures generated as described above in Example 1 and subjected to hemodynamic conditions. RNA was isolated from tumors cells grown for 2, 4, and 7 days under hemodynamic conditions. This comparison indicated that transcriptosome of the tri-cultures subjected to hemodynamic conditions more closely resembled the *in vivo* xenografts than the *in vitro* static 2D cultures.

[00411] RNA-seq data were generated using a reverse strand library preparation from A549 NSCLC cells grown tri-cultures as described above in Example 1 and as illustrated i FIG. 9, under the three different matrix configurations. Approximately 20 million 50 bp paired-end reads were sequenced per sample. Raw sequence reads were aligned to the University of California Santa Cruz (UCSC) annotations of known isoforms in the hg19 assembly of the human genome using the Bowtie aligner (Langmead et al. 2009). Estimates of read counts per isoform were computed using the eXpress tool (Roberts and Pachter, 2012). Gene-wise counts were generated by summing the estimated counts across isoforms for each gene. Genes with low counts across the entire experiment were not considered in downstream analyses. Specifically, genes that were detected at a level of at least 2 counts per million in fewer than 5 samples were discarded. After filtering genes with low signal, library sizes were normalized using the Trimmed Mean of M-values (TMM) method (Robinson and Oshlack, 2010).

[00412] To distinguish human from mouse RNA in the xenograft samples, we used a strategy similar to that employed in Raskatov et al., 2012. Raw reads were aligned to the human hg19 and mouse mm10 transcriptomes simultaneously. The alignments were then quantified for each transcript (human and mouse) using eXpress. All eXpress estimates computed for mouse transcripts were discarded, and estimates for human transcripts were used in downstream analyses.

[00413] Gene-wise differential expression analysis was performed on the filtered and TMM-normalized abundance estimates using edgeR (Robinson et al., 2010). The NSCLC gene set is from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al., 1999). The gene set consists of: AKT3, CDK4, CDK6, RASSF1, E2F1, E2F2, E2F3, EGF, EGFR, ERBB2, AKT1, AKT2, EML4, GRB2, HRAS, ARAF, KRAS, NRAS, PDPK1, PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PLCG1, PLCG2, PRKCA, PRKCG, MAPK1, MAPK3, MAP2K1, MAP2K2, RAF1, RARB, CCND1, RXRA, RXRB, SOS1, SOS2, BRAF, TGFA, RASSF5, PIK3R3, FOXO3, BAD, CASP9, RB1, STK4.

[00414] The results of RNA-seq transcriptosomal profiling are shown in Figures 16–18. Figure 16 provides a dendogram (FIG. 16A) and a heatmap (FIG. 16B) showing the clustering of samples based on the gene-centered log2 expression values of all 14,159 genes that reach the signal threshold. A549 cells grown in the tri-cultures generated as described above and subjected to hemodynamic conditions clustered based on the matrix conditions. "CL" = collagen layer; "CS" = collagen sandwich; NC = no collagen. Xenograft samples

clustered separately, with the exception of the no collagen condition on day 7, suggesting that increased time in the device in the absence of collagen more faithfully recapitulates the transcriptomic profile of xenograft tissue, relative to the other culture conditions. The hierarchical clustering was performed using an average linkage criteria, and Spearman rank correlation as the distance metric.

[00415] Figure 17 provides a dendogram (FIG. 17A) and a heatmap (FIG. 17B) showing the clustering of samples based on the log2 fold changes of the 7935 genes differentially expressed between the xenograft and plastic conditions at a 5% false discovery rate (FDR). Xenograft samples cluster with the samples from tri-cultures subjected to hemodynamic conditions that do not contain exogenous collagen. This indicates that the transcriptomic differences between xenograft and static two-dimensional cultures are better reproduced in the absence of exogenous collagen. The hierarchical clustering was performed using an average linkage criteria, and Spearman rank correlation as the distance metric. "CS" = collagen sandwich; "NC" = no collagen; "plastic" = static 2D cultures.

[00416] Figure 18 provides a dendogram (FIG. 18A) and a heatmap (FIG. 18B) showing the clustering of samples based on the log2 fold changes (condition versus static 2D cultures ("plastic")) of the 48 genes in this dataset that are annotated with "Non-small cell lung cancer" in the KEGG database. Xenograft samples cluster with the samples from tri-cultures subjected to hemodynamic conditions that do not contain collagen. This indicates that the transcriptional responses of genes that are highly relevant to the disease phenotype are more similar to xenografts in the absence of collagen than in the presence of collagen. The hierarchical clustering was performed using an average linkage criteria, and Spearman rank correlation as the distance metric. CS = collagen sandwich; NC = no collagen; "plastic" = static 2D cultures. The 48 genes in the data set, the expression of which is indicated in the rows of the heatmap, from top the top row to the bottom row are: EGF, RXRB, CASP9, CDK4, RASSF5, PRKCA, FOXO3, RARB, PIK3R2, MAPK3, RXRA, AKT2, GRB2, ARAF, MAP2K2, AKT1, RASSF1, HRAS, BRAF, EML4, STK4, NRAS, MAPK1, RAF1, MAP2K1, PDPK1, PIK3CB, TGFA, BAD, PLCG2, E2F2, E2F1, PRKCG, EGFR, CDK6, PLCG1, ERBB2, PIK3R3, SOS2, PIK3R1, KRAS, SOS1, PIK3CA, CCND1, PIK3CD, E2F3, AKT3, and RB1.

Example 8: Molecular Activity Profiling of Endothelial Cells and Tumor Cells

[00417] In *in vivo* tumor-vasculature regions, tumor cell proliferation and invasion is modulated by endothelial cells in close proximity to the tumor cells. Qualitative and quantitative gene expression and cytokine secretion profiles for both the endothelial cells and the tumor cells cultured according to the methods described in Example 1 can be generated. Such assays are used to monitor the expression of angiogenic and tumorigenic factors and demonstrate that endothelial cells cultured under tumor-derived hemodynamic flow alter the molecular signature of the tumor cells, e.g., by enhancing markers of tumor molecular activity as measured using next generation mRNA sequencing and cytokine and growth factor secretion profiling.

[00418] After establishing tri-cultures as described in Example 1 above, mRNA can be collected from endothelial and tumor cells for analysis. Table 1 lists genes in both endothelial and tumor cells that are known to be regulated in the tumor microenvironment *in vivo*. This panel of genes serves as an initial molecular assessment of the tri-cultures described in Example 1 to demonstrate that the heterotypic cell-cell communication and hemodynamic flow and transport in the coculture systems affect the molecular activity of the tumor microenvironment. Table 1 (right) also lists a series cytokine and growth factors that are profiled using a multiplexing platform (MAGPIX), which is capable of performing qualitative and quantitative analysis of angiogenic factors such as VEGF and angiopoietins. Because of the design of the system, media can be collected in real-time from the endothelial cell and tumor cells layers separately for analysis.

Table 1.

Gene	*Function*	mRNA		MAGPIX	
		EC	A549	EC	549
VEGFA	Angiogenesis	✓	✓	✓	✓
VEGFC	Angiogenesis	✓	✓	✓	✓
VEGFD	Angiogenesis	✓	✓	✓	✓
ANG1	Angiogenesis	✓	✓		
ANG2	Angiogenesis	✓	✓	✓	✓
FGF-2	Angiogenesis		✓		✓
PLGF	Angiogenesis		✓		✓
EGF	Proliferation	✓	✓	✓	✓
EGFR	Proliferation		✓		
MKI67	Proliferation	✓	✓		
PCNA	Proliferation	✓	✓		
VIM	Invasion		✓		

CDH1	Invasion		✓		
CDH2	Invasion		✓		
IL-6	Inflammation	✓	✓		
IL-8	Inflammation	✓	✓	✓	✓
NF-kB	Inflammation	✓			
eNOS	Inflammation	✓			
KLF2	Inflammation	✓			
MCP-1	Inflammation	✓	✓	✓	✓

[00419] After reproducing the baseline molecular activity with a minimum of five biological replicates, next-generation sequencing-based mRNA transcriptomics (RNAseq) can be performed on the endothelial cells and tumor cells cultured as described above in Example 1, and compared to the following controls: (1) a static co-culture system, (2) endothelial cells subjected to tumor-derived shear stress in the absence of tumor cells, (3) tumor cells in monoculture; and (4) *in vivo* tumors. The OncoMine Research database is used to compare and contrast these results against >73,000 cancer expression profiles obtained from a large variety of human tumor samples, including clinical outcomes in >27,000 samples, pathway/drug responses in >7,800 samples and >11,000 samples from the Cancer Genome Atlas (TCGA). This provides an unbiased assessment of the coculture tumor microenvironment system.

Example 9: Testing of Anti-Cancer Drugs in In Vitro *Tumor Microenvironment Tri-Cultures*

[00420] The effects of anti-cancer drugs on the A549 tumor cells grown in tri-cultures generated as described above in Example 1 and depicted in FIG. 9 were assessed. The following drugs were selected: cisplatin, a front line chemotherapeutic for NSCLC (Rossi et al., 2012; National Cancer Institute, Non-Small Cell Lung Cancer Treatment (PDQ®)), and two experimental small molecule allosteric inhibitors of MEK (AZD6244/Selumetinib) and AKT (MK-2206), which are in clinical trials for NSCLC and other cancers (Leijen et al., 2011; National Cancer Institute, Randomized Phase II Study of ADZ6244; Yap et al., 2011; National Cancer Institute, MK2206 and Erlotinib Hydrochloride in Treating Patients with Advanced Non-Small Cell Lung Cancer). For each of the three drugs tested in the tri-cultures subjected to hemodynamic conditions, growth inhibition occurred at the clinically relevant human patient C_{max}.

[00421] The IC_{50}s of these three drugs for A549 tumor cells grown under static 2D conditions does not approximate the clinically relevant doses used in cancer patients. For

cisplatin the *in vivo* C_{max} is 3μM and thus represents the clinical dose that patients with NSCLC receive (Urien et al., 2005). However, the cisplatin IC_{50} for A549 cells in static 2D cultures from published results ranges from 6μM to over 60μM (Andriani et al., 2006; Zhang et al., 2013; Zhang et al., 2003; Barr et al., 2013). Thus, cisplatin is routinely *in vitro* used at 2X to 20X higher concentrations than what is achievable *in vivo* for NSCLC patients. Similarly, the A549 IC_{50} for AZD6244/Selumetinib and MK2206 in static 2D cultures is significantly higher than the dose achieved in patients (Yeh et al., 2007; Meng et al., 2010). For AZD6244/Selumetinib the C_{max} is 1.4μM and the static 2D IC_{50} is 5μM, a 3.5-fold increase. For MK2206 the C_{max} is 160nM and the static 2D IC50 is 3μM, nearly a 19-fold increase. These differences in C_{max} and static 2D IC_{50}s help illustrate a major barrier that confronts work in static 2D conditions; non-physiologic drug concentrations are frequently necessary for biologic effects.

[00422] Table 2 summarizes the human and mouse in vitro C_{max}s and the *in vitro* IC50s for static cultures for cisplatin, MK2206, and AZD6422, as well the concentrations of the drugs tested tri-cultures subjected to hemodynamic conditions.

Table 2.

	Cisplatin	**MK2206**	**AZD6422**
Human C_{max}	3 μM	160 nM	1.4 μM
Mouse C_{max}	>10 μM	540 nM	5.5 μM
Static 2D IC_{50}	>6 μM	3 μM	5 μM
Concentration applied to tri-cultures subjected to hemodynamic conditions	3 μM	160 nM	1.4 μM

[00423] The IC_{50} concentrations of cisplatin, MK2206 and AZD6244/selumetinib for the A549 cell line listed in Table 2 were taken from the literature as described above and estimated to be >6 μM, 3 μM and 5 μM, respectively. Maximal plasma concentrations (C_{max}) in mice were estimated using pharmacokinetic data from taken from efficacy studies in the peer-reviewed literature. For cisplatin, the steady state plasma C_{max} was determined to be >10 μM (Johnsson et al., 1995). For MK2206, the steady state plasma C_{max} was determined to be 540 nM (Piovan et al., 2013). For AZD6244/selumetinib, the steady state plasma C_{max} was determined to be 5.5 μM (Denton and Gustafson, 2011). Maximal plasma concentrations (C_{max}) in humans were estimated with pharmacokinetic data from clinical trials using established therapeutic dosing paradigms. For cisplatin, the steady state plasma C_{max} was determined to be 3 μM (Salas et al., 2006; Urien et al., 2005). For MK2206, the steady state

plasma C_{max} was determined to be 160 nM (Hudis et al., 2013; Yap et al., 2011). For AZD6244/selumetinib, the steady state plasma C_{max} was determined to be 1.4 μM (Adjei et al., 2011; O'Neil et al., 2011).

[00424] The growth rate of the human lung carcinoma cell line A549 was determined in response to vehicle control ("Veh"), cisplatin, MK-2206, and AZD6244 using the human C_{max} doses described above. Tri-cultures were prepared as described above in Example 1 and as depicted in FIG. 9 in the absence of exogenously added ECM and subjected to tumor capillary hemodynamics. The vehicle control or drug was added to the inflow media for the endothelial cell layer and perfused into the upper volume. Tri-cultures were maintained in the presence of absence of drugs for up to seven days under hemodynamic flow and transport. Cell number was determined at day seven using the QUANT-IT PICOGREEN dsDNA Assay Kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY). Data are presented in FIGs. 19A and 19B as the mean number of cells from duplicate.

[00425] FIG. 19A shows the cell number for A549 tumor cells treated with the vehicle control ("Veh"), cisplatin, MK2206, or AZD6422. FIG. 19B shows the relative cell growth of A549 tumor cells treated with the vehicle control ("Veh"), cisplatin, MK2206, or AZD6422. Cisplatin inhibited A549 growth by 48%, MK2206 by 44%, and AZD6422/Selumetinib by 78%. These data indicate that tumor cells grown in the tri-cultures subjected to hemodynamic conditions respond to both established chemotherapeutics (cisplatin) and experimental small molecule inhibitors (MK2206, AZD6442) at therapeutically relevant concentrations.

[00426] Collectively, these data indicate that tumor cells grown in the tri-cultures and subjected to hemodynamic conditions respond to both established chemotherapeutics and experimental small molecule inhibitors at human patient physiologic doses. It is important to note that as shown in Table 2, the mouse C_{max} at therapeutic doses for cisplatin, AZD6244/Selumetinib and MK2206 is higher than the human patient Cmax. In mice the C_{max} of AZD6244/Selumetinib is 5.5μM (Meng et al., 2010, Chung et al., 2009), which is equivalent to the static 2D IC_{50}. For MK2206 the mouse Cmax is 540nM (Meng et al., 2010), which is 5-fold higher than the human patient C_{max}. Cisplatin has a mouse C_{max} ranging from 10–100μM (Andriani et al., 2006; Zhang et al., 2003; Bain et al., 2007), which exceeds the human patient C_{max} and is equivalent to or higher than the static 2D IC_{50}. Thus, the tri-

cultures subjected to hemodynamic conditions may be superior to mouse xenograft studies for testing experimental drugs at physiologic doses.

Example 10: Further Testing of Anti-Cancer Drugs in In Vitro *Tumor Microenvironment Tri-Cultures*

[00427] Additional anti-cancer drugs can be tested in for their effects on the tumor cells, stromal fibroblasts, and/or microvascular endothelial cells grown in tri-cultures generated as described above in Example 1. The anti-cancer drug can be introduced into the tri-cultures at concentrations in the culture medium that are within the concentration range of the *in vivo* therapeutic C_{max} in a human. For example, the antiproliferative chemotherapeutic drug carboplatin (Sigma-Aldrich), and the EGFR inhibitor erlotinib (Cayman Chemical) can be tested. These two drugs are commonly used in the treatment of lung cancer, their pharmacokinetics have been well-characterized through clinical trials, and they both have been shown to improve the survival of lung cancer patients. Carboplatin is a traditional chemotherapeutic drug that indiscriminately kills rapidly dividing cells, whereas erlotinib is a targeted therapy that is not as broadly toxic.

[00428] The drug can be added to the inflow media for the endothelial cell layer and perfused into the upper volume. Initially, the drugs can be applied to the endothelial cells at two doses, with the highest dose being the *in vivo* C_{max} level achieved in humans (for carboplatin, 37 μg/ml; for erlotinib, 1 μg/ml), and a 10-fold lower dose, mimicking the lower degree of drug penetration observed in many solid tumors *in vivo*. A broader range of doses can then used to generate response profiles for each drug. As a two-dimensional control, A549 cells can be plated on tissue culture-treated dishes and cultured in conditioned media from microvascular endothelial cells in the presence of each drug, and cell counts can be performed after 3 days. As a three-dimensional control, A549 cells can be seeded in a three-dimensional collagen matrix on the bottom of a well of a 6-well dish, and endothelial cells plated on the upper surface of the porous membrane of a cell culture insert inserted into the well, similar to current *in vitro* three-dimensional tumor models. The endpoints described in the Examples above can be used to assess the effect of the anticancer agent, e.g. cell viability, tumor cell invasion, endothelial cell permeability, RT-PCR, mRNA sequencing, and cytokine and growth factor profiling.

[00429] Additional anti-cancer drugs can be tested in a similar manner and their dose-response curves compared with the dose-response curves for the drug in *in vivo* models.

Such drugs include, for example, angiogenesis inhibitors such as the VEGF inhibitor bevacizumab and the VEGFR inhibitor sorafenib.

Example 11: A Model for Tumor Metastasis to the Liver

[00430] The metastasis of tumor cells through the vasculature to distal organs is the major cause of mortality in cancer. The liver is a common site for tumor metastases because if its rich blood supply. Human tumor cells (e.g., the A549 tumor cells or pancreatic tumor cells) are cultured according to the methods described above in Examples 1, under static 2D conditions, or as xeongrafts. The tumor cells are then extracted and added to an *in vitro* liver model system, for example, an *in vitro* system liver model system as described in U.S. Patent Publication No. US 2013/0309677 and PCT Publication No. 2013/0158939, the contents of both of which are hereby incorporated by reference in their entirety. For example, in an *in vitro* liver model system described in U.S. Patent Application Publication No. US 2013/0309677 and PCT Publication No. 2013/0158939, hepatocytes are sandwiched in a collagen gel and plated on a first surface of a porous membrane. Sinusoidal endothelial cells are optionally plated on the second surface of the porous membrane. Additional non-parenchymal cells (e.g., hepatic stellate cells, Kupffer cells, or a combination thereof) are optionally plated on the first or second surface of the porous membrane. A shear stress that mimics *in vivo* blood flow in the liver is then applied to the non-parenchymal cells on the second side of the porous membrane, and cell culture medium is perfused into and out of the upper and lower volumes. Figure 11 provides a schematic drawing of such a system.

[00431] Tumor cells are extracted from the tri-cultures, xenografts, or 2D static cultures and added to this *in vitro* liver system. The tumor cells are either introduced directly into the lower volume containing the hepatocytes, or to the upper volume optionally containing the sinusoidal endothelial cells and/or other non-parenchymal cells. In the latter case, transmigration of the tumor cells into the lower volume containing the hepatocytes is assessed. Growth of the tumor cells in the *in vitro* liver system is also assessed.

[00432] Alternatively, a coculture system containing tumor cells, stromal fibroblasts, and microvascular endothelial cells as described above in Example 1 is linked to an *in vitro* liver model system by tubing that is used to transfer culture medium from the lower or upper volume of the tumor model coculture to the lower or upper volume of the *in vitro* liver model system as shown in Figures 12A–D. This creates a model of tumor metastasis mimicking the seeding of distal organs by tumor cells *in vivo*.

[00433] Efficacy of anticancer agents can be assessed by measuring the growth of these *in vitro* metastases in the *in vitro* liver model in the presence of an anticancer agent, as compared to the growth of the in vitro metastases in the absence of the anticancer agent.

Example 12: A Physiologic In Vitro *Liver Model*

[00434] Static hepatocyte cell culturing methods are associated with poor *in vitro* to *in vivo* correlations, due in part to the absence of physiological parameters which maintain metabolic phenotype over time *in vivo*. Restoring physiological hemodynamics and transport retains hepatocyte phenotype and function *in vitro* compared to the standard static hepatocyte collagen gel configuration.

[00435] To recreate a cellular hepatocyte system with fluid dynamics and transport analogous to *in vivo* liver circulation, a cone-and-plate device-based technology was employed that has been extensively used to re-establish *in vivo* blood vessel cell phenotypes by recreating the exposure of vascular endothelial cells to human-derived hemodynamic blood flow forces *in vitro*. This technology is described in U.S. Patent No. 7,811,782. The technology was adapted and modified to design a rat liver monoculture system which applies hemodynamic flow and transport conditions reflective of *in vivo* hepatic circulatory values. The configuration of cells in the device is based on *in vivo* microarchitecture of hepatic lobules where cords of hepatocytes are separated from sinusoidal blood flow by a filtering layer of endothelial cells. This design uses a porous polycarbonate membrane suspended in a cell culture container, with primary rat hepatocytes sandwiched in a collagen gel on one side of the porous membrane. The porous membrane acts analogously to the filtering layer of sinusoidal endothelial cells which is present in the liver. Media is continuously perfused on both sides of the porous membrane, while hemodynamic forces, derived from a range of physiological blood flow values, are continuously applied to the non-cellular side of the porous membrane. The entire set up is housed in a controlled environment with 5% CO_2 and at 37°C. A flow-based culture system was effectively created whereby hepatocytes are shielded from direct effects of flow, as they would be *in vivo*. Recapitulating the hemodynamics and in a system designed to be analogous to the microstructure of the hepatic sinusoid results in stable retention of a differentiated hepatic and metabolic phenotype similar to that of *in vivo* liver.

METHODS

(i) Animal Surgery and Hepatocyte Isolation

[00436] All animals used for the experiments were treated according to protocols approved by HemoShear's Animal Care & Use Committee. Hepatocytes were isolated from male Fischer rats (250g–350g) by a modification of Seglen's two-step collagenase perfusion procedure using a 20 mL/min flow rate (Seglen, *Hepatocyte Suspensions and Cultures as Tools in Experimental Carcinogegnesis, J. Toxicology & Environmental Health*, 5(2–3): 551–560 (1979), the contents of which are hereby incorporated by reference). Briefly, the rats were anaesthetized with isoflurane, following which the abdominal cavity was incised and the inferior vena cava was canulated while making an excision was made in the portal vein for outflow. The liver was perfused in two steps, first with a Ca^{++}-free buffer to flush out blood and break up intercellular junctions, followed by collagenase in a Ca^{++}-containing buffer to digest the extracellular collagen matrix. After the liver was suitably perfused it was excised and freed of the capsule in a Petri dish under a sterile hood. An enriched hepatocyte population (~95% purity) was obtained by two sequential 65g centrifugation and washing cycles of 10 minutes each followed by a 10 minute spin with 90% PERCOLL (colloidal silica particles of 15–30 nm diameter (23% w/w in water) coated with polyvinylpyrrolidone (PVP); used to establish density gradients that can be used to isolate cells). The viability of hepatocytes was determined by trypan blue exclusion test and cells with a viability over 85% are used.

(ii) Cell Culture and Device Operating Conditions

[00437] *Hepatocyte Culture Media*: For the data shown in Figures 20–24, the rat hepatocyte culture media contained base media of DMEM/F12 containing high glucose (17.5 mM), supplemented by fetal bovine serum (10% at the time of plating and reduced to 2% for maintenance after 24 hours). The media also contained gentamycin (50 μg/ml), ITS (insulin concentration 2 μMol), 1% NEAA, 1% GLUTAMAX, and dexamethasone (1 μM at plating and 250 nM for maintenance after 24 hours).

[00438] For the data shown in Table 6 and Figures 38 and 39, the rat hepatocyte culture media contained base media of DMEM/F12 containing low glucose (5.5 mM), supplemented by HEPES (3% vol/vol) and fetal bovine serum (10% vol/vol at the time of plating and reduced to 2% for maintenance after 24 hours). The media also contained

gentamycin (50 μg/ml), ITS (insulin concentration 2 nMol), 1% NEAA, 1% GLUTAMAX, and dexamethasone (1 μM at plating and 100 nM for maintenance after 24 hours).

[00439] To culture human or dog hepatocytes, the culture media contained base media of DMEM/F12 containing low glucose (5.5 mM), supplemented by HEPES (3% vol/vol) and fetal bovine serum (10% vol/vol at the time of plating and reduced to 2% for maintenance after 24 hours). The media also contained gentamycin (50 μg/ml), ITS (insulin concentration 2 nMol), and dexamethasone (1 μM at plating and 100 nM for maintenance after 24 hours).

[00440] *Collagen coating and plating*: Collagen solution was made by mixing Type I Rat Tail Collagen in sterile distilled water, 10X phosphate buffered saline (PBS) and 0.2N sodium hydroxide in a predefined ratio (To make up 1 ml, the components were 440 μl, 375μl, 100μl and 85μl respectively).

[00441] For cultures to be subjected to static conditions, 100 mm tissue culture-treated sterile cell culture dishes were coated with 7 $\mu l/cm^2$ of collagen solution. For cultures to be subjected to controlled hemodynamics, the lower surface of the porous membrane of 75 mm TRANSWELLS (polycarbonate, 10 μm thickness and 0.4 μm pore diameter, no. 3419, Corning) were coated with 7 $\mu l/cm^2$ of collagen solution. After allowing an hour for the solution to gel, the surfaces were washed with DPBS, hepatocytes were plated at a seeding density of 125,000 viable cells/cm^2, and a second layer of collagen gel added after 4 hours. After 1 hour, the TRANSWELLS were inverted and placed into cell culture dishes, and media was added (9 ml in the lower volume and 6 ml in the upper volume). 7 ml of media was added to the tissue culture dishes to be used for static cultures. After 24 hours, the media was switched to maintenance media (containing 2% FBS), and the cell culture dishes containing TRANSWELLS were placed into the cone-and-plate device. Controlled hemodynamics were applied to the surface of the porous membrane of the TRANSWELL in the upper volume.

[00442] Cryopreserved human hepatocytes were procured from commercial vendors (Kaly-Cell, France) and thawed as per the vendor's prescribed protocols. For plating human hepatocytes, a similar procedure to that described above for rat hepatocytes was followed, a limited cell-seeding area was used. The second layer of collagen was applied as described above.

[00443] Freshly isolated canine hepatocytes from beagle dogs were procured from commercial vendors (Triangle Research Laboratories, Research Triangle Park, North

Carolina) and processed as per the vendor's prescribed protocols. For plating canine hepatocytes, a similar procedure to that described above for rat hepatocytes was followed, but using a limited cell-seeding area. The second layer of collagen was applied as described above.

[00444] *Operating conditions*: The shear stress in dynes/cm^2 (τ) was calculated for a typical hepatic sinusoid based on the formula for pressure driven flow of a Newtonian fluid through a cylinder,

$$\tau = \frac{\Delta P . r}{2l}$$

using reference values for pressure gradient across the sinusoid (ΔP), radius of sinusoids (r) and length of the sinusoids (l) from the literature. As part of an initial optimization process, a range of applied shear stress conditions obtained by altering media viscosity and cone speed that resulted in rates within an order of magnitude of the value predicted from literature were seen to be correlated with different transport profiles of horse radish peroxidase dye across the membrane. These were tested for gene expression profiles of the hepatocytes 7 days into culture (data not shown). No differences were observed between static cultures and those that were simply perfused without any applied shear and based on the gene expression profiles, an operational shear rate of 0.6 dynes/cm^2 was selected for all the experiments described in this Example.

(iii) Assessment of Phenotypic, Functional, Metabolic, and Toxic Parameters

[00445] *RT-PCR:* Changes in metabolic, toxic, and insulin/glucose/lipid pathway genes were assessed by extracting RNA from hepatocytes from devices run under healthy and steatotic conditions at the end of the culture period (7 or 14 days) and performing RT-PCR on this RNA. The TRANSWELLS were removed from the devices and washed with PBS prior to scraping the cells off the porous membrane. Total RNA was isolated using a PURELINK RNA Mini Kit (a kit for purification of total RNA from cells) and reverse transcribed to cDNA using the ISCRIPT cDNA Synthesis Kit (a cDNA synthesis kit). Primers were designed for the metabolic genes CYP1A1, CYP1A2, CYP3A2, MDR, and GST as well as the insulin/glucose/lipid pathway genes GPAT, ACC1, IRS-2, PPAR-γ, SREBP, ChREBP, LXR, SCD1, CPT1. Primer sequences are shown below in Table 3:

Table 3: Rat Primer Sequences

Gene	Forward (SEQ ID NO.)	Reverse (SEQ ID NO.)
CYP1A1	GCTGCTCTTGGCCGTCACCA (1)	TGAAGGGCAAGCCCCAGGGT (2)
CYP1A2	CCTGCGCTACCTGCCCAACC (3)	GGGCGCCTGTGATGTCCTGG (4)
CYP3A2	CGGCGGGATTTTGGCCCAGT (5)	CAGGCTTGCCTGTCTCCGCC (6)
MDR	GCTGCTGGGAACTCTGGCGG (7)	CCGGCACCAATGCCCGTGTA (8)
GST (Pi subunit)	CGCAGCAGCTATGCCACCGT (9)	CTTCCAGCTCTGGCCCTGGTC (10)
GPAT	AGCGTTGCTCCATGGGCATATAGT (11)	TGTCAGGGATGGTGTTGGATGACA (12)
ACC1	TGTCATGGTTACACCCGAAGACCT (13)	TTGTTGTTGTTTGCTCCTCCAGGC (14)
IRS-2	GCGAGCTCTATGGGTATATG (15)	AGTCCTCTTCCTCAGTCCTC (16)
PPAR-g	ATATCTCCCTTTTTGTGGCTGCTA (17)	TCCGACTCCGTCTTCTYGATGA (18)
SREBP	GGAGCCATGGATTGCACATT (19)	AGGCCAGGGAAGTCACTGTCT (20)
ChREBP	CTATGTCCGGACCCGCACGC (21)	CTATGTCCGGACCCGCACGC (22)
LXR	ACTCTGCAACGGAGTTGTGGAAGA (23)	TCGGATGACTCCAACCCTATCCTT (24)
SCD1	TGTGGAGCCACAGGACTTACAA (25)	AGCCAACCCACGTGAGAGAAGAAA (26)
CPT1	ATGTGGACCTGCATTCCTTCCCAT (27)	TTGCCCATGTCCTTGTAATGTGCG (28)
CYP2B1	GAGGAGTGTGGAAGAACGGATTC (29)	AGGAACTGGCGGTCTGTGTAG (30)
CYP2B2	TCATCGACACTTACCTTCTGC (31)	AGTGTATGGCATTTTGGTACGA (32)
SORD	TCTGTGGCTCGGATGTTCACTACT (33)	CGGCCGATCTTGCAGAATTCATCT (34)
GSR	GGACTATGACAACATCCCTACC (35)	CCAACCACCTTCTCCTCTTT (36)
APEX1	GCCTAAGGGCTTTCGTTACA (37)	ATCCACATTCCAGGAGCATATC (38)
MRP3	AGGCCAGCAGGGAGTTCT (39)	AGCTCGGCTCCAAGTTCTG (40)
MRP4	CAACTCCTCTCCAAGGTGCT (41)	ATCTGCTCACGCGTGTTCTT (42)

[00446] RNA expression was analyzed by real-time RT-PCR using IQ SYBR Green Supermix (a PCR reagent mixture for RT-PCR) and a CFX96 Real-Time System with C1000 Thermal Cycler (an RT-PCR detection system and thermal cycler). RNA data were normalized to endogenous expression of β2-microglobulin and reported as a relative quantity compared to healthy cultures.

[00447] Human genes assessed for metabolism and toxicity experiments included CYP1A1. CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP3A4, CYP3A5, GSTA1, UGT1A1, GSR, SORD, TXNRD1, and APEX1. The primer sequences for these are shown in Table 4. Canine genes assessed for metabolism included CYP1A1 and CYP3A12 (primer sequences shown in Table 5).

Table 4: Human Primer Sequences

Gene	Forward (SEQ ID NO.)	Reverse (SEQ ID NO.)
CYP1A1	GGACCTGAATGAGAAGTTCTACAGC (43)	AGCTCCAAAGAGGTCCAAGACGAT (44)
CYP2A6	TCATAGCCAAGAAGGTGGAGCACA (45)	CCCAATGAAGAGGTTCAACGTGGT (46)
CYP2B6	GGGCACACAGGCAAGTTTACAA (47)	AGAGCGTGTTGAGGTTGAGGTTCT (48)
CYP2C9	TGACTTGTTTGGAGCTGGGACAGA (49)	ACAGCATCTGTGTAGGGCATGT (50)
CYP2D6	ACGACACTCATCACCAACCTGTCA (51)	AGGTGAAGAAGAGGAAGAGCTCCA (52)
CYP3A4	CTGCATTGGCATGAGGTTTGCTCT (53)	AAATTCAGGCTCCACTTACGGTGC (54)
CYP3A5	CTGCATTGGCATGAGGTTTGCTCT (55)	AGGGTTCCATCTCTTGAATCCACC (56)
GSTA1	GATGCCAAGCTTGCCTTGAT (57)	AGGGAAGCTGGAGATAAAGACTGGA (58)
UGT1A1	GGCCCATCATGCCCAATATGGTTT (59)	GCATCAGCAATTGCCATAGCTTTC (60)
SORD	TAGCGCCACCAGAAGCGACCAAA (61)	TCATTTGGGCCTGGTTCAGGGATA (62)
APEX1	CCAGCCCTGTATGAGGACC (63)	GGAGCTGACCAGTATTGATGAGA (64)
GSR	CACTTGCGTGAATGTTGGATG (65)	TGGGATCACTCGTGAAGGCT (66)
TXNRD1	ATATGGCAAGAAGGTGATGGTCC (67)	GGGCTTGTCCTAACAAAGCTG (68)

Table 5: Canine Primer Sequences

Gene	Forward (SEQ ID NO.)	Reverse (SEQ ID NO.)
CYP1A1	CACCATCCCCCACAGCACAACAAA (69)	GCTCTGGCCGGAATGCAAATGGAT (70)
CYP3A12	GAGAGAATGAAGGAAAGTCGCC (71)	GCCACCAGCTCCAAATCAGA (72)
B2MG	TCCTCATCCTCCTCGCT (73)	TTCTCTGCTGGGTGTCG (74)

[00448] *Urea and Albumin Assays:* Media collected from static cultures and devices at various time points was assayed for albumin using a rat-specific ELISA based kit (Bethyl Laboratories) as per the manufacturer's protocols. Urea was estimated from the media samples using a standard colorimetric assay (QUANTICHROM Urea Assay Kit, DIUR-500, Gentaur). All measurements between the systems were normalized to a per million cells/day rate for comparison based on the volume of media perfused and the number of initially plated cells.

[00449] *Western Blots:* Following application of controlled hemodynamics, 1/3 of the plated surface of the porous membrane of the TRANSWELL (~1.8 million cells) was harvested for protein in 150 μl 1X RIPA buffer containing fresh 150 mM DTT and protease inhibitors (HALT Protease Inhibitor Cocktail (Pierce) + 1mM PMSF + 200 mM DTT). Samples were sonicated on ice with 5 × 1 second pulses, allowed to sit on ice for 30 minutes and centrifuged at 17,000×g for 10 minutes in a chilled microcentrifuge. Protein determination was done using A660 nm Protein Reagent (Pierce). Samples were boiled 70°C for 10 minutes and then run on a 7.5% TGX gel (a pre-cast polyacrylamide gel, BioRad) before wet-transferring to 0.2 μm PVDF membrane and blocking in 5% non-fat milk at room

temperature for 10 minutes. Membranes were incubated overnight at 4°C in rabbit anti UGT antibody (Cell Signaling, 1:500 dilution). Secondary antibody (Santa Cruz, Goat anti Rabbit HRP, 1:5000 dilution) incubation was at room temperature for one hour. Chemiluminescent signal was developed using SUPERSIGNAL WEST PICO (a chemiluminescent substrate for horseradish peroxidase, Pierce) reagent and captured using an Innotech ALPHAEASE imaging system. For normalization, gels were probed for mouse anti β-Actin (Sigma A1978, 1:2000 dilution) followed by secondary goat anti mouse HRP (Santa Cruz sc-2005, 1:10,000 dilution).

[00450] *Immunostaining and Biliary Activity Stain:* Antibodies used: Hnf4a (Santa Cruz sc-8987), E-cadherin (Santa Cruz sc-71009), and anti-MRP2 (Abcam ab3373). At the chosen time points in the experimental design, the static cultures and cultures subjected to controlled hemodynamics were washed gently with 1x PBS, following which they were fixed with 4% paraformaldehyde for 30 minutes. The samples were stored in PBS at 4°C until they were to be immunostained. For immunostaining, the samples were first permeabilized with 0.1% TRITON X (a nonionic surfactant) for 20 minutes and then washed with PBS and blocked with 5% goat serum. The incubation with primary antibodies was at a dilution of 1:100 for 1 hour. After 3 washes with PBS with 1% BSA, the secondary antibody was added at a dilution of 1:500 for another hour. The samples were then washed with PBS plus 1% BSA and then mounted for confocal imaging.

[00451] For imaging of the biliary activity at canalicular junctions, sections of the porous membrane of the TRANSWELL were washed with PBS and incubated with media containing 10 μM carboxy-2,7-dichlorofluorescein diacetate (CDFDA) for 10 minutes. Samples were then washed with PBS and placed on glass slide for confocal imaging.

[00452] *Transmission Electron Microscopy*: Transmission electron microscopy was performed as described below in Example 13.

[00453] *Cytochrome Activity Assays:* Hepatocytes were cultured in the cone-and-plate devices under static or controlled hemodynamic conditions for five days, and then treated with 0.1% dimethyl sulfoxide (DMSO) or known inducers of cytocrhome enzymes(3-methylcholanthrene and dexamethasone) for 48 hours. Porous membrane segments roughly $2cm^2$ in area were excised and transferred to standard 24-well plates alongside corresponding static cultures. The cells were incubated with 500 μl of hepatocyte media containing substrates from commercially available P450-GLO kits (kits for luminescent cytochrome p450 assays) at the manufacturer-recommended concentrations. After 4 hours, the media was

transferred to 96-well plates and assayed for luminescent metabolites to reflect cytochrome p450 activity as per the manufacturer protocol. The ATP content of the cells in the same porous membrane segments or static wells was then estimated by the CELLTITER-GLO assay (a kit for a luminescent cell viability assay) using the manufacturer's protocol, and the cytochrome values were normalized to ATP content.

[00454] To assess CYP activity and induction responses of human hepatocytes, the cells were plated and cultured in the cone-and-plate devices and subjected to controlled hemodynamics under the operating conditions described above or were cultured under static conditions (controls) for 7 days before being exposed to either 0.1% DMSO or known CYP inducer drugs phenobarbital (500 μM for static and 50 μM for devices) or rifampicin (25 μM for static and 2.5 μM for devices) for 72 hours. The hepatocytes were then incubated with medium containing a cocktail of CYP substrates [(ethoxy resorufin (10 μM), midazolam (3 μM), bufuralol hydrochloride (10 μM), (S)-mephenytoin (50 μM), bupropion hydrochloride (100 μM), and diclofenac sodium (10 μM)] for 4 hours. The culture supernatants were then collected and analyzed by HPLC for formation of metabolites to assess specific activity of specific CYP enzymes. All values were normalized to protein content of the cells.

[00455] *Gluconeogenesis Assays*: Primary rat hepatocytes isolated and plated as described above were cultured in the cone-and-plate devices under controlled hemodynamics for 7 days. Hepatocytes were washed with PBS and incubated in glucose free media, with addition of substrates glycerol (2 mM) or lactate (20 mM) and pyruvate (2 mM) in the presence or absence of the regulatory hormones insulin (2 nM) or glucagon (100nM). After 4 hours, the supernatants were collected and assayed for glucose content using the colorimetric AMPLEX RED kit (a glucose/glucose oxidase assay kit, Life Technologies) as per manufacturer's instructions. The glucose values were normalized to the protein content of the cellular lysates.

[00456] *MTT Assay*: To assess toxicity responses of human hepatocytes, the cells were plated and cultured in the cone-and-plate devices under hemodynamic conditions using the operating conditions described above or were cultured under static conditions (controls) for 7 days before being exposed to either 0.1% DMSO or known toxic drug chlorpromazine (0.1 μM, 1 μM and 10 μM) for 72 hours. Hepatocytes were then incubated with medium containing 1mg/ml of MTT reagent (thiazolyl blue tetrazolium bromide) for 1 hour, following which the cells were lysed in DMSO to release the formazan blue dye formed. The solution was transferred to a 96 well plate and the absorbance was read at 595 nm.

[00457] *Live-Dead Staining*: To assess toxicity responses of human hepatocytes, the cells were plated and cultured in the cone-and-plate devices under hemodynamic conditions under the operating conditions described above for 7 days or were cultured under static conditions (controls) before being exposed to either 0.1% DMSO or known toxic drug chlorpromazine (0.1 μM, 1 μM and 10 μM) for 72 hours. At the end of the treatment period, the hepatocytes were washed with PBS and then incubated in LIVE/DEAD viability/cytotoxicity reagent (Invitrogen) at a concentration of 2 μM calcein AM and 4 μM ethidium homodimer-1 (EthD-1) for 30 minutes. Cells were then mounted between glass coverslips and imaged using a confocal microscope.

[00458] *miRNA122 assay*: Rat hepatocytes were plated and cultured in the cone-and-plate devices under controlled hemodynamic or were cultured under static conditions (controls) using the operating conditions described above for 7 days. The hepatocytes were then washed with PBS and incubated with serum free hepatocyte medium with or without known toxic drug chlorpromazine (CPZ) at two different concentrations (1 μM and 10 μM) for 4 hours. Supernatants from the cells were collected and microRNA extraction was performed using the MIRNEASY serum/plasma kit (a kit for extracting microRNA, Qiagen). The cDNA was prepared by using the MISCRIPTII RT kit (a kit for preparing cDNA, Qiagen) and samples quantified using the MISCRIPT SYBR GREEN PCR kit (a kit for quantifying cDNA, Qiagen), following the manufacturer's instructions.

RESULTS

(ii) *Controlled hemodynamics maintain hepatocyte phenotype, polarized morphology and transporter localization relative to traditional static monoculture conditions.*

[00459] Freshly isolated rat primary hepatocytes were obtained and plated in collagen gel sandwiches on porous membranes. After 1 day, cultures were either continued under standard static conditions in a CO_2 incubator at 37°C or introduced into the hemodynamic flow technology and maintained under controlled hemodynamics at pre-determined indirect shear rates of 0.6 dynes/cm^2. Media was changed every 48 hours in static cultures and the devices were continuously perfused. After 7 days, the cultures were removed and fixed with 4% paraformaldehyde before immunostaining with antibodies for the hepatocyte differentiation markers E-cadherin and HNF-4α, and visualized by confocal microscopy. E-cadherin staining patterns in static collagen gel sandwich cultures (Figure 20A) displayed higher levels of cytoplasmic E-cadherin confirmed and quantified by

morphometric analysis (adjacent graphs) and disrupted peripheral membrane distribution. Under controlled hemodynamics (Figure 20B), hepatocytes exhibited a more differentiated morphology characterized by distinct peripheral membrane localization and lower cytoplasmic levels of E-cadherin. The staining pattern of the HNF4α showed a distinct difference in localization patterns with the cells in static cultures having a more diffuse staining pattern by 7 days (Figure 20C) while the cells under controlled hemodynamics retained staining confined to the nucleus (Figure 20D), similar to what is seen *in vivo*. Polarized morphology and canalicular localization of the transporter multi drug resistant protein -2 (MRP-2) that appears after 5–7 days of culture in collagen gel sandwiches is lost in static cultures by day 14 (Figure 20E) but the canalicular network patterns are stable and extensive under controlled hemodynamics (Figure 20F). Day 14 cultures maintained under controlled hemodynamics co-stained for MRP-2 and HNF-4α (Figure 21A) alongside sections from rat *in vivo* liver (Figure 21B) show very similar staining patterns. Transmission electron microscopy images of day 7 cultures under controlled hemodynamics (Figure 21C) demonstrate the retention of subcellular components such as rough and smooth endoplasmic reticulum and mitochondria in addition to confirming the presence of bile canaliculi and tight junctions.

(ii) Controlled hemodynamics results in retention of hepatocyte-specific function in rat hepatocytes in a collagen gel configuration relative to static cultures over 14 days.

[00460] Hepatocytes were cultured under static or controlled hemodynamics (0.6 dynes/cm^2) for 2 weeks and media sampled at 4, 7, 11, and 14 days. Assays for urea and albumin were performed on the media and the values were normalized to production rates over 24 hours per million cells based on the initial number of plated cells. Hepatocyte function reflected by secreted albumin estimated from media samples at various time points over 14 days and expressed as μg/10^6 plated hepatocytes/day (Figure 22A), showed significantly higher levels (3–4 fold) under controlled hemodynamics (solid line) as compared to static cultures (dashed line) (Day 7: 97.96±11.34 vs. 25.84±8.22, p = 0.00001; Day 14: 87.80±8.62 vs. 33.93± 4.39, p= 0.0001). Urea secretion (Figure 22B) by hepatocytes expressed as μg/10^6 plated hepatocytes/day under controlled hemodynamics (solid line) was also found to be at 4–5 fold higher levels than static cultures (dashed line) consistently over two weeks in culture (Day 7: 622.78±33.96 vs. 139.76±13.37, p = 2.7 x 10^{-9}; Day 14: 667.71±84.37 vs. 178.68± 6.13, p= 1 x 10^{-6}).

(iii) Controlled hemodynamics differentially regulates the expression of phase I and phase II metabolic genes and proteins compared to static cultures.

[00461] Hepatocytes were cultured under static or controlled hemodynamics (0.6 dynes/cm^2) for 7 days. QRT-PCR was performed for select metabolic genes (Table 3) on RNA samples at day 7 from these conditions. All values were normalized to day 7 static cultures. Hepatocytes cultured under controlled hemodynamics resulted in gene expression levels that were consistently higher than in static cultures (n=11, Fold changes relative to static cultures: Cyp1A1 ~54, p = 0.0003; Cyp1A2 ~ 64, p = 0.005, Cyp2B1 ~15, p = 0.001: Figure 23A, Cyp2B2 ~ 2.7, p=0.09 and Cyp3A2 ~ 4, p=0.075: Figure 23B) and closer to *in vivo* levels. Interestingly, the expression levels of the gene for the Pi subunit of phase II enzyme GST, known to increase in static cultures over time, was lower in both *in vivo* liver (–4.9 fold, p = 0.152) and hepatocytes cultured under controlled hemodynamics (–2.3 fold, p = 0.025) compared to static cultures (Figure 23C).

[00462] Hepatocytes were cultured under static or controlled hemodynamics (0.6 dynes/cm^2). Cell cultures were taken down at 4,7, 11 and 14 days and cell lysates were obtained as described in the methods section, normalized to total protein, and equivalent samples were loaded and run on SDS page gels before probing with antibodies for the phase II enzyme UGT1 A1 and β-actin (for normalization). Western blots (Figure 23D) demonstrate that UGT1 A1 is upregulated under controlled hemodynamics as compared to static conditions at all the time points over 2 weeks in culture. In the same experiment, part of the porous membrane of the TRANSWELL from 14 day cultures under controlled hemodynamics was fixed with 4% paraformaldehyde and stained for HNF-4a and the canalicular transporter protein MRP-2, demonstrating retention and localization of MRP-2 along the canalicular junctions between the hepatocytes (Figure 21A). The remainder of the membrane was excised after removal from the device and immediately incubated with the substrate carboxy-2,7-dichlorofluorescein diacetate (CDFDA). The cells were imaged by confocal microscopy over a time window of 20 minutes to observe the breakdown of the substrate into carboxy-2,7-dichlorofluorescein (CDF) and its active secretion into the bile canalicular structures (seen in Figure 21C). The pattern was very similar to that of sectioned samples of *in vivo* liver immunostained with antibodies to MRP-2 and HNF-4a (Figure 21B).

(iv) Rat hepatocytes cultured under controlled hemodynamics display a higher level of basal and inducible cytochrome p450 activity than static cultures at more in vivo-like concentrations.

[00463] To validate that the increase in metabolic genes and proteins translated to changes in metabolic activity, primary rat hepatocytes were cultured as described earlier in the cone-and-plate devices under controlled hemodynamics (0.6 dynes/cm^2) and in static collagen gel cultures. After 5 days, they were either left untreated or treated with 0.1% DMSO, 1A/1B inducer 3-Methyl Cholanthrene (3-MC, 1μM in static and 0.1μM under controlled hemodynamics) or 3A inducer dexamethasone (50μM in static and 02.5μM under controlled hemodynamics). After 48 hours, on day 7, segments of the porous membrane from the devices containing hepatocytes cultured under controlled hemodynamics that were roughly 2.0 cm^2 in area were excised and transferred to standard 24-well plates and treated with substrates for the Cyp p450 enzymes in parallel to corresponding static cultures treated with the different agents. Cytochrome p450 assays were done on day 7 using commercially available P450-GLO kits. After 4 hours the media was transferred to 96-well plates and assayed for luminescent metabolites to reflect cytochrome p450 activity. Values were normalized to the ATP content of the cells assessed by CELLTITER-GLO assay in order to get an accurate representation of live cells and avoid any confounding effects of the collagen gels on total protein measurements.

[00464] Basal activity level of the cytochrome p450 enzymes (Figure 24A) in untreated cultures was upregulated by controlled hemodynamics compared to static (1A ~ 15 fold, 1B ~ 9 fold and 3A ~ 5 fold). In spite of higher levels of basal activity, under controlled hemodynamics the response to classical inducers (Figure 24B) was well maintained (1A/1B response to DMSO vs. 3-MC - 4.87 vs. 133.06; 3A response to DMSO vs. Dexamethasone – 11.64 vs. 57.53).

[00465] While initially measuring the Cyp activity to confirm the enhanced gene expression that was noted under controlled flow, 50 μM dexamethasone, the concentration recommended for inducing static cultures, was toxic in this system. As a result the concentration of the dexamethasone was decreased to 1 μg/ml in order to get an inductive response, a level that correlates well with plasma concentrations seen *in vivo* in rats. Similarly, induction responses for 3-MC were also seen at 10-fold lower levels under controlled hemodynamics.

[00466] To confirm the presence of transporter activity under controlled hemodynamics, TRANSWELL filter segments from the devices were incubated with the substrate carboxy-2,7-dichlorofluorescein diacetate (CDFDA). The compound was broken down to the fluorescent form CDF Carboxy-2,7-Dichlorofluorescein which was actively secreted out into the canalicular spaces demonstrating active canalicular transport (Figure 24C).

[00467] The data described above are the result of experiments carried out to evaluate the effect of exposing hepatocytes to controlled hemodynamics in order to restore their phenotype more similar to that observed *in vivo*. These experiments used standard media formulations routinely used in static culture in order to allow for side by side comparison with the static collagen gel cultures and identify the selective benefits of controlled hemodynamics. In the course of these experiments, hepatocytes cultured under these controlled hemodynamic conditions demonstrated enhanced *in vivo*-like phenotype and function and were more responsive to inducers such as dexamethasone and 3-MC. However, some accumulation of lipids was also observed in hepatocytes cultured with the concentrations of glucose (17.5 mM) and insulin (2 μMol) which are used routinely for assays in static systems. It was discovered that when hepatocytes are cultured under controlled hemodynamic conditions as described herein, much lower concentrations of glucose and insulin, similar to the concentrations observed in healthy individuals *in vivo*, can be used. The data indicate that these lower concentrations of glucose (5.5 mM) and insulin (2 nM) further enhance hepatocyte function and metabolic activity. Moreover, hepatocytes can be cultured under controlled hemodynamics in media containing the higher concentrations of glucose and insulin in order to create a model of fatty liver disease, as explained further in the following Example.

(v) Primary rat hepatocytes cultured under controlled hemodynamics demonstrate responsiveness to insulin and glucagon.

[00468] Primary rat hepatocytes isolated and plated as described above were cultured in the cone-and-plate devices under controlled hemodynamics for 7 days prior to washing with PBS and incubation with the substrates glycerol (2 mM) or lactate (20 mM) and pyruvate (2 mM) either in the presence or absence of the regulatory hormones insulin (2 nM) or glucagon (100 nM). Glucose levels measured in the supernatant after 4 hours by the AMPLEX RED assay showed that in the absence of a substrate, insulin decreased glucose

levels by 27% while glucagon increased it by 51%. In the presence of the substrate glycerol, glucose produced by the hepatocytes increased by 67%. Addition of glucagon increased glucose levels by further 15% while insulin decreased glucose levels by 38%. When lactate and pyruvate were used as substrates, glucose produced by the hepatocytes increased in the presence of glucagon by 80% while insulin decreased glucose levels by 25%. These data are summarized in Table 6.

Table 6.

Substrate	Effect of Insulin (% Change)	Effect of Glucagon (% Change)
No substrate	– 27%	+ 51%
Glycerol (+ 67%)	– 38%	+ 15%
Lactate/Pyruvate	– 25%	+ 80%

(vi) Cryopreserved human hepatocytes cultured under controlled hemodynamics demonstrate induction responses to phenobarbital and rifampicin at in vivo *level concentrations.*

[00469] Human hepatocytes were cultured in the cone-and-plate devices under controlled hemodynamics under the operating conditions described above or were cultured under static conditions (controls) for 7 days before being exposed to the known CYP inducer drugs phenobarbital (500 μM in for static conditions and 50 μM for controlled hemodynamic conditions) or rifampicin (25 μM in for static conditions and 2.5 μM for controlled hemodynamic conditions) for 72 hours. The hepatocytes were then washed with PBS and incubated with medium containing a cocktail of CYP substrates as described above for 4 hours. The culture supernatants were then collected and analyzed for formation of metabolites to assess specific activity of specific CYP enzymes. Results were normalized to protein content of the cells and expressed as pmol/min/mg of protein. Vehicle treated controls with DMSO 0.1% exhibited higher levels of CYP2B6, CYP2C9 and CYP3A4 in under controlled hemodynamic conditions as compared to static conditions (7.7 vs. 4.6, 4.6 vs. 0.5 and 7.6 vs. 0.7 pmol/min/mg of protein, respectively). Treatment with phenobarbital at the lower concentration (50 uM) under controlled hemodynamic conditions compared to higher concentration under static conditions (500 μM) also resulted in comparable or higher levels of enzyme activities of CYP2B6, CYP2C9 and CYP3A4 (45.9 vs. 34.3, 16.3 vs. 0.9 and 16.3 vs.

3.8 pmol/min/mg of protein, respectively). Similarly, treatment with rifampicin at the lower concentration (2.5 μM) under controlled hemodynamic conditions compared to the higher concentration in static conditions (25 μM) also resulted in comparable or higher levels of enzyme activities of CYP2B6, CYP2C9 and CYP3A4 (87.3 vs. 131.1, 1.4 vs. 16.0 and 11.5 vs. 23.1 pmol/min/mg of protein, respectively). These results are depicted in Figure 35.

(vii) Cryopreserved human hepatocytes cultured under controlled hemodynamics demonstrate toxicity responses to chlorpromazine at in vivo *level concentrations.*

[00470] Cryopreserved primary human hepatocytes thawed and plated as described above were cultured in the cone-and-plate devices under controlled hemodynamics or were cultured under static conditions (controls) for 7 days before being exposed to different concentrations of chlorpromazine (0.1 μM, 1 μM, and 10 μM) or vehicle control for 72 hours. Live-dead staining was performed on the hepatocytes with ethidium-calcein stain. Hepatocytes were also incubated with MTT reagent for 1 hour to assess viability. RNA was extracted from additional segments and RT-PCR was performed to assess selected toxicity and metabolic genes. Hepatocytes cultured under static conditions did not exhibit any toxicity at all the concentrations tested. However hepatocytes cultured under controlled hemodynamics demonstrated dose-dependent toxicity with 30.3% toxicity at 1 μM and 46.4% toxicity at 10 μuM (Figure 36B). At 1 μM, the toxicity to the hepatocytes cultured under controlled hemodynamics devices was also detected by live-dead staining (Figure 36A).

[00471] RT-PCR demonstrated upregulation of various oxidative stress related toxicity genes at 1 μM chlorpromazine under controlled hemodynamic conditions relative to static controls (8.3-fold for glutathione reductase (GSR), 5.5-fold for thioredoxin reductase 1 (TXNRD1), 6.9-fold for sorbitol dehydrogenase (SORD), and 2.8-fold for APEX nuclease (multifunctional DNA repair enzyme)). Concomitantly, certain metabolic genes were also upregulated under controlled hemodynamic conditions relative to static controls (17.8-fold for cytochrome p450 family 1 member A2 (CYP1A2), 8.4-fold for cytochrome p450 family 1 member A1 (CYP1A1), and 5.6-fold for Cytochrome p450 family 2 member B6 (CYP2B6). These results are depicted in Figure 37. The results shown in Figure 37 used primary human hepatocytes from KalyCell Donor #B0403VT.

[00472] These data show that primary human hepatocytes display toxic responses to chlorpromazine at clinical plasma C_{max} concentrations under controlled hemodynamic

conditions. These toxic responses are associated with the upregulation of oxidative stress-related genes and certain metabolic genes.

(viii) Primary rat hepatocytes cultured under controlled hemodynamics demonstrate acute toxicity and release miRNA122 in response to chlorpromazine exposure at in vivo *level concentrations.*

[00473] Primary rat hepatocytes isolated and plated as described above were cultured in the cone-and-plate devices under controlled hemodynamic conditions or were cultured under static conditions (controls) for 7 days. The hepatocytes were washed with PBS and immediately incubated with either vehicle (distilled water) or chlorpromazine (1 μM) for 4 hours. The supernatant was collected and miRNA 122 levels were measured as described above. It was seen that under static conditions, chlorpromazine at 1 μM did not cause any change in miRNA 122 levels in the supernatants compared to vehicle controls. By contrast, hepatocytes cultured under controlled hemodynamic conditions and incubated with chlorpromazine (1 μM) for 4 hours released miRNA at significantly higher levels (6-fold over vehicle controls). These results are depicted in Figure 38.

(ix) Primary rat hepatocytes cultured under controlled hemodynamics demonstrate sublethal toxicity and exhibit cholestatic changes in response to troglitazone exposure at in vivo *level concentrations.*

[00474] Primary rat hepatocytes isolated and plated as described above were cultured in the cone-and-plate devices under controlled hyemodynamic conditions for 5 days before being exposed to 4 μM or 40 uM troglitazone for 48 hours. The hepatocytes were washed with PBS and and immediately incubated with the substrate 10 uM carboxy-2,7-dichlorofluorescein diacetate (CDFDA). The cells were imaged by confocal microscopy during a 20-min exposure to the nonfluorescent substrate CDFDA to allow for the hydrolysis of the substrate to the highly fluorescent Mrp-2 substrate carboxy-2,7-dichlorofluorescein (CDF) and its active secretion into the bile canalicular structures. At 4 uM, troglitazone was found to cause changes in the canalicular pattern with visibly dilated canalicular structures. These changes were much more prominent and extensive at 40 uM troglitazone (Figure 39). The toxic response of rat hepatocytes to troglitazone at *in vivo*/clinical plasma C_{max} concentrations when cultured under controlled hemodynamic conditions was associated with

upregulation of oxidative stress-related genes and compensatory upregulation of MRP3 and MRP4 genes (Figure 40).

(x) Primary dog hepatocytes cultured under controlled hemodynamics demonstrate retention of polarized morphology and exhibit higher expression of key metabolic genes relative to static cultures.

[00475] Freshly isolated canine hepatocytes were cultured in the cone-and-plate devices under controlled hemodynamic conditions under operating conditions similar to those described above for human hepatocytes or were cultured under static conditions (controls). After 7 days, cultures were fixed and stained with phalloidin and Draq5 for actin cytoskeleton and nucleus, respectively. RNA was collected from cells and RT-PCR was performed for specific metabolic genes. Canine hepatocytes were seen to retain polarized morphology with polygonal shape at 7 days and to express CYP1A1 and CYP3A12 at significantly higher levels than static controls (6.7- and 7.4-fold respectively). These results are depicted in Figure 41.

Example 13: An In Vitro *Model for Fatty Liver Disease*

[00476] Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver dysfunction and is associated with obesity, insulin resistance, and type 2 diabetes. The changes in the fatty liver progress from early accumulation of fat vesicles within hepatocytes (hepatic steatosis) to subsequent loss of liver metabolic function and inflammatory changes, ultimately leading to fibrosis and cirrhosis. Animal *in vivo* models of fatty liver disease have successfully used either high fat diets or low fat, high carbohydrate diets that induce the hyperglycemia and hyperinsulinemia reflective of the diabetic milieu to induce triglyceride buildup. However *in vitro* models typically use only overloading with free fatty acids (oleic, palmitic or linoleic acid) to induce fatty changes and may not capture the *de novo* hepatocyte response to the high levels of glucose and insulin that may play a critical role in the pathogenesis of the disease. Static hepatocyte cultures are also known to have a markedly decreased insulin response and standard culture medias typically require high non-physiological levels of the hormone for basic hepatocyte survival and function. The model described herein, by contrast, preserves a more physiological hepatocyte response to drugs and hormones and allows us to maintain basic liver function at closer to *in vivo* concentration levels of glucose and insulin (as described above in Example 12), and furthermore allows us

to elicit the pathologic response seen in fatty liver by creating a diabetic-like milieu characterized by high glucose and insulin levels.

METHODS:

(i) Animal Surgery and Hepatocyte Isolation

[00477] Animal surgery and hepatocyte isolation were performed as described above in Example 12.

(ii) Cell culture and Device Operating Conditions

[00478] *Healthy hepatocyte culture media*: The healthy hepatocyte culture media contained base media of DMEM/F12 containing low glucose (5.5 mM), supplemented by fetal bovine serum (10% at the time of plating and reduced to 2% for maintenance after 24 hours). Additionally, the media contained gentamycin (50 μg/ml), ITS (insulin, transferrin, and selenium; insulin concentration of 2 nM), 1% non-essential amino acids (NEAA), 1% GLUTAMAX (a media supplement containing L-alanyl-L-glutamine), and dexamethasone (1μM at plating and 250nM for maintenance after 24 hours for the data shown in Figures 25 and 26; 100 nM throughout the experiment for the data shown in Figures 27–34).

[00479] *Media to induce fatty liver changes ("fatty liver media")*: The culture media used to induce fatty liver changes contained base media of DMEM/F12 containing high glucose (17.5 mM), supplemented by fetal bovine serum (10% at the time of plating and reduced to 2% for maintenance after 24 hours). The media also contained gentamycin (50 μg/ml), ITS (insulin concentration 2 μMol), 1% NEAA, 1% GLUTAMAX, and dexamethasone (1μM at plating and 250nM for maintenance after 24 hours for the data shown in Figures 25 and 26; 100 nM throughout the experiment for the data shown in Figures 27–34).

[00480] *Collagen coating and plating*: Collagen solution was made as described above in Example 12. The lower surfaces of the porous membranes of 75 mm TRANSWELLS (polycarbonate, 10 μm thickness and 0.4 μm pore diameter, no. 3419, Corning) were coated with 300 μl of the collagen solution. After allowing an hour for the solution to gel, the surfaces were washed with DPBS, hepatocytes were plated at a seeding density of 125,000 viable cells/cm^2, and a second layer of collagen gel added after 4 hours. After 1 hour, the TRANSWELLS were inverted and placed into cell culture dishes, and media was added (9 ml in the lower volume and 6 ml in the upper volume). After 24 hours

(i.e., on day 2 of the experiments), the media was changed to maintenance media (the healthy or fatty liver media described above) and the Petri dishes were placed in the cone-and-plate hemodynamic flow device, and controlled hemodynamics were applied to the surface of the porous membrane of the TRANSWELL in the upper volume. In some experiments, the maintenance media contained 1.5 μM pioglitazone in 0.1% DMSO vehicle or the 0.1% DMSO vehicle alone. The cells were cultured under controlled hemodynamics until day 7, when hepatocytes were examined using the assays described below.

[00481] *Operating conditions*: The shear stress was calculated as described above in Example 12. A range of applied shear stress conditions, generated by altering media viscosity and cone speed, and resulting in rates within an order of magnitude of the value predicted from literature (0.1 to 6 dynes/cm^2) were used. These were correlated with different transport profiles of reference dye horse radish peroxidase dye across the membrane. Cultures were run for 7 days and assessed for fatty liver changes.

(iii) Measurement of Fatty Liver Changes:

[00482] To examine changes occurring in the fatty liver model against healthy controls the following were evaluated:

(a) Changes in metabolic and insulin/glucose/lipid pathway genes (RT-PCR);
(b) Accumulation of intracellular lipids within hepatocytes by Oil Red O assay, Nile red staining, and measurement of total triglycerides;
(c) Changes in differentiated function of hepatocytes (urea and albumin secretion);
(d) Changes in metabolic activity (Cytochrome p450 assays); and
(e) Morphological changes within hepatocytes by transmission electron microscopy (TEM).

[00483] RT-PCR and urea and albumin assays were performed as described above in Example 12.

[00484] *Staining Methods*: Hepatocyte TRANSWELL membrane sections were permeabilized in 0.1% Triton-X diluted in PBS for 20 minutes and washed thrice in PBS for five minutes each. Samples were then blocked in 5% goat serum, 0.2% blotting grade non-fat dry milk blocker, and 1% BSA in PBS for 45 minutes. The samples were then washed thrice in 0.1% BSA in PBS and incubated with 1:5000 dilution of Nile red (1mM stock), 1:1000 DRAQ5 (a fluorescent DNA dye; Cell Signalling), 1:500 ALEXA FLUOR 488 conjugated

phalloidin (Life Technologies), and 1% BSA in PBS for thirty minutes and protected from light. The samples were washed in 0.1% BSA in PBS thrice for five minutes each and mounted on glass cover slips using PROLONG GOLD antifade mounting media (an antifade reagent; Invitrogen). The samples were imaged on a Nikon C1+ Confocal System microscope.

[00485] *Transmission Imaging Microscopy (TEM):* Segments of the porous membranes from TRANSWELLS containing hepatocytes cultured under healthy or steatotic conditions for 7 days were washed with PBS before fixing in a solution containing 4% paraformaldehyde and 2% glutaraldehyde for 1 hour. The samples were then sent to be processed for TEM at the University of Virginia imaging center. TEM images were evaluated for accumulation of lipid within the hepatocytes, the appearance of subcellular organelles such as mitochondria and smooth and rough endoplasmic reticulum, retention of polarized morphology, and bile canaliculi.

[00486] *Oil Red O Assay:* Accumulation of intracellular lipids within hepatocytes was assessed by adapting and modifying a commercially available Steatosis Colorimetric Assay Kit (Cayman Chemical). At the end of the culture period, 2 cm^2 sized porous membrane segments containing the hepatocytes from devices under healthy and steatotic conditions were washed with PBS and fixed in 4% paraformaldehyde for 30 minutes. These porous membrane segments were then washed with PBS, dried completely and incubated with 300 µl of Oil Red O working solution for 20 minutes in 24 well plates. The porous membrane segments were then washed repeatedly with distilled water 7–8 times followed by two five minute washes with the wash solution provided in the Steatosis Colorometric Assay Kit. Dye extraction solution (300 µl) was added to each well and the plates were incubated on an orbital shaker for 15–30 minutes under constant agitation. The solution was then transferred to clear 96-well plates and absorbance was read at 490–520 nm in a spectrophotometer.

[00487] *Measurement of Total Triglycerides:* Triglyceride content was assessed using a commercially available colorimetric assay kit (Cayman Triglyceride Colorimetric Assay Kit, Cat # 10010303). At the end of the treatment period, cells were collected from the porous membranes by scraping with a rubber policeman and PBS, after which they were centrifuged (2,000 x g for 10 minutes at 4°C). The cell pellets were resuspended in 100 µl of cold diluted Standard Diluent from the triglyceride assay kit and sonicated 20 times at one second bursts. The cell suspension was then centrifuged at 10,000 x g for 10 minutes at 4°C.

The supernatant was removed and used for the assay as per the manufacturer's protocol and normalized to protein content from the same samples.

[00488] *Cytochrome Activity Assays:* Hepatocytes were cultured in the cone-and-plate devices under healthy and steatotic conditions for 7 days. Porous membrane segments roughly $2cm^2$ in area were excised and transferred to standard 24-well plates alongside corresponding static cultures. The cells were incubated with 500 µl of healthy hepatocyte media containing substrates from commercially available P450-GLO kits at the manufacturer-recommended concentrations. After 4 hours, the media was transferred to 96-well plates and assayed for luminescent metabolites to reflect cytochrome p450 activity as per the manufacturer protocol. The ATP content of the cells in the same porous membrane segments or static wells was then estimated by the CELLTITER-GLO assay using the manufacturer's protocol, and the cytochrome values were normalized to ATP content.

RESULTS:

[00489] *Nile red staining*: Figures 27A and B show staining of hepatocytes cultured in the healthy (Fig. 27A) or fatty liver (Fig. 27B) media with Nile red, phalloidn, and DRAQ5. As can be seen in Figure 27B, the hepatocytes cultured in the fatty liver media (containing high concentrations of glucose and insulin) accumulate a large number of lipid droplets.

[00490] *Transmission electron microscopy*: Hepatocytes cultured in the fatty liver media were also examined by transmission electron microscopy. As shown in Figure 28, hepatocytes cultured under these conditions accumulate lipid. A large lipid droplet is indicated in the hepatocyte on the left side of the image. Gap junctions between two hepatocytes are also shown, demonstrating the polarized morphology.

[00491] *Total lipid and total triglycerides*: As shown in Figure 29, total lipid (Fig. 29A) and total triglycerides (Fig. 29B) were both significantly increased in hepatocytes cultured under the high glucose/high insulin fatty liver conditions in the presence of liver-derived hemodynamics. Oil red O quantification indicated that the total lipid was raised in the disease cultures by about 3-fold as compared to the healthy cultures.

[00492] *Gene expression*: Glycerol 3-phosphate acyltransferase (GPAT) is a key enzyme involved in triglyceride synthesis and known to upregulated and contribute to steatosis and fatty liver. As shown in Figure 25, primary rat hepatocytes cultured under controlled hemodynamics in the devices when exposed to pathological conditions (n=9) of

high insulin (2 μMol) and high glucose (17.5 mMol) exhibit a significantly higher expression the GPAT gene (p=0.04) compared to those cultured under healthy physiological levels (n=6) of insulin (2 nMol) and glucose (5.5 mMol) in the media. The results are expressed as fold increase over standard static cultures in collagen gel sandwiches (2 μMol insulin and 17.5 mMol glucose).

[00493] Similar results are shown in Figure 30B for hepatocytes cultured under controlled hemodynamics in healthy or fatty liver media containing a lower concentration of dexamethasone. The hepatocytes cultured in the high insulin/high glucose (fatty liver) media exhibited significantly higher levels of GPAT expression as compared to hepatocytes cultured in the healthy media containing lower levels of insulin and glucose. As shown in Figure 30A, hepatocytes cultured under controlled hemodynamics in the high insulin/high glucose media also exhibited significantly higher levels of expression of sterol regulatory element-binding protein (SREBP), another key gene responsible for lipogenisis, as compared to hepatocytes cultured in the healthy media.

[00494] These steatotic changes were accompanied by concomitant metabolic changes. Of all the key metabolic enzymes, the cytochrome p450 3A family is responsible for the metabolism of a majority of drugs. As shown in Figure 26, primary rat hepatocytes cultured under controlled hemodynamics in the devices with healthy physiological levels (n=6) of insulin (2 nMol) and glucose (5.5 mMol) in the media, exhibit a significantly higher expression level of the key metabolic enzyme cytochrome p450 3a2 (Cyp3A2; p=0.03), compared to those cultured under pathological conditions (n=9) with high insulin (2μMol) and high glucose (17.5 mMol) levels. Both the healthy and pathological fatty liver levels under controlled flow are many fold higher than static cultures in collagen gel sandwiches (2μM insulin and 17.5 mMol glucose).

[00495] Similarly, as shown in Figure 31A, expression of a number of phase I enzymes involved in drug metabolism are differentially regulated under low and high glucose/insulin conditions. Under hemodynamic flow, hepatocytes under healthy media conditions maintained high levels of mRNA expression of Cyp1a1, Cyp 2b1, 2b2, Cyp3a2,and (20, 90, 30 and 40-fold higher than traditional static cultures respectively), whereas Cyp 2b2 and Cyp 3a2 levels in hepatocytes cultured in the fatty liver media were decreased by 9 and 12 fold compared to healthy.

[00496] *Cyp Activity*: As shown in Figure 31B, the activities of CYP3A2 and CYP1A1 were also reduced 3–6-fold under the high insulin/glucose fatty liver conditions compared to healthy, as measured by the p45glo assay.

[00497] *Pioglitazone treatment*: Pioglitazone, a drug used to treat steatosis, was tested in the fatty liver model to determine if it could reverse the lipid accumulation and metabolic changes induced by the high insulin/glucose fatty liver media. The pioglitazone was added to the media at a concentration of 1.5 μM, a concentration selected based on the therapeutic C_{max} observed for pioglitazone *in vivo*. Pioglitazone was effective in reducing the lipid buildup and triglyceride content while restoring metabolic gene expression under the disease conditions. As shown in Figure 32, Nile red staining indicates that treatment with pioglitazone at *in vivo* therapeutic concentrations decreases lipid droplet formation under steatotic conditions. Pioglitazone also reduced total triglyceride content of hepatocytes cultured in the high insulin/glucose media to levels similar to those seem in the hepatocytes cultured under healthy conditions (Figure 33). Moreover, as shown in Figure 34, pioglitazone restored the expression of metabolic genes such as Cyp3A2 which are depressed by the high insulin/glucose disease conditions.

CONCLUSIONS:

[00498] In summary, a system was developed that preserves *in vivo*-like hepatocyte phenotype and response, to create a model of hepatic steatosis by inducing pathological steatotic changes in the presence of a high glucose/insulin milieu. Rat hepatocytes under controlled hemodynamics retain their response to insulin and glucose, and hepatocytes cultured under hemodynamic flow develop steatotic changes when cultured in high glucose and insulin ('disease') conditions. The steatosis is mediated via *de novo* lipogenesis with upregulation of two key genes (SREBP and GPAT), and the increase in lipid accumulation and triglyceride content is accompanied by a concomitant decrease in metabolic gene expression and activity. Treatment with the PPAR-γ agonist pioglitazone helps prevent the buildup of lipid and loss of metabolic activity under the high glucose and insulin conditions. These data demonstrate a novel and important new *in vitro* model of diet induced non-alcoholic fatty liver disease (NAFLD) for which none currently exist.

Example 14: An inducible pluripotent stem cell (iPSC)-derived human hepatocyte system

[00499] Hepatocytes derived from inducible pluripotent stem cells (iPSCs) offer a potential solution for eliminating variability and studying genotypic variation in drug response but have not found widespread acceptance on account of the fetal phenotype and inadequate metabolic profile they exhibit in standard, static culture systems. The data described above in Example 12 demonstrate that primary rat and human hepatocytes, which are known to rapidly dedifferentiate under static culture conditions, stably retain a mature differentiated phenotype when cultured under controlled hemodynamic conditions, resulting in a more physiologic drug and hormone response. It was discovered that iPSCs respond similarly when physiological properties such as flow, hemodynamics and transport are maintained and exhibit the differentiated liver phenotype and response to drugs that they exhibit *in vivo*.

METHODS

(i) iPSC-derived Hepatocytes

[00500] iPSC-derived Hepatocytes were purchased from Cellular Dynamics International.

(ii) iPSC-derived Hepatocyte Culture Media

[00501] The iPSC-derived hepatocyte culture media for static cultures was as per the vendors recommendations. For cells cultured under controlled hemodynamic conditions in the cone-and-plate devices, a base media of Williams E medium supplemented by fetal bovine serum (10%) and dexamethasone (1 μM) at the time of plating was used. Maintenance media was used after 24 hours that did not contain FBS but was supplemented with bovine serum albumin (0.125%). The media also contained gentamycin (25 μg/ml), ITS (insulin concentration 2 nMol), 1% NEAA, 1% GLUTAMAX, HEPES (30mM) and dexamethasone (100nM).

(iii) Collagen coating and plating

[00502] The collagen coating and plating conditions were identical to those described above in Example 12 for primary human hepatocytes. The iPSC-derived hepatocytes were dissociated and plated as per the vendor's protocols using the recommended media. iPSC-derived hepatocytes were cultured under static conditions or were transferred

into the cone-and-plate devices after 24 hours for further culture under controlled hemodynamic conditions.

RESULTS

(i) Hepatocytes derived from inducible pluripotent stem cells (iPSCs) cultured under controlled hemodynamic conditions retain polarized morphology and exhibit higher expression of key metabolic genes relative to static cultures.

[00503] iPSC-derived hepatocytes cultured in the cone-and-plate devices under controlled hemodynamics for 10 days retain polarized morphology (Figure 42) and exhibit higher expression of key metabolic genes relative to static cultures (104-fold for CYP1A1, 91-fold for CYP1A2, 8.8-fold for CYP3A4, 8.2-fold for CYP2B6, 2.3-fold for CYP2C9 and 2.3-fold for CYP2D6). Expression of the constitutive androstane receptor CAR was 6.0-fold higher than cells cultured under static conditions and the liver-specific protein albumin was at 2.2-fold higher levels than in cells cultured under static conditions. These results are depicted in Figure 43.

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[00539] When introducing elements of the present invention or the preferred embodiments(s) thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[00540] In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained.

[00541] As various changes could be made in the above methods without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

[0100] WHAT IS CLAIMED IS:

1. A method for mimicking a tumor microenvironment *in vitro*, the method comprising:

adding a culture medium to a cell culture container;

plating at least one tumor cell type on a surface within the cell culture container; and

indirectly applying a shear stress upon the at least one tumor cell type, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment, wherein the flow is time-variant.

2. The method of claim 1, further comprising:

depositing at least one extracellular matrix component on the surface within the cell culture container and plating the at least one tumor cell type on the at least one extracellular matrix component; or

suspending the at least one tumor cell type in a solution comprising at least one extracellular matrix component to create a suspension comprising the at least one tumor cell type and the at least one extracellular matrix component, and depositing the suspension on the surface within the cell culture container; and

indirectly applying the shear stress upon the at least one extracellular matrix component and the at least one tumor cell type.

3. The method of claim 1, further comprising plating the at least one tumor cell type on a first surface of a porous membrane and indirectly applying the shear stress upon the at least one tumor cell type by applying the shear stress upon a second surface of the porous membrane.

4. A method for mimicking a tumor microenvironment *in vitro*, the method comprising:

adding a culture medium to a cell culture container;

plating at least one tumor cell type on a first surface of a porous membrane within the cell culture container; and

indirectly applying a shear stress upon the at least one tumor cell type by applying a shear stress upon a second surface of the porous membrane, the shear stress resulting from

flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

5. A method for mimicking a tumor microenvironment *in vitro*, the method comprising:

adding a culture medium to a cell culture container;

plating at least one tumor cell type or stromal cell type on a first surface of a porous membrane within the cell culture container, wherein when the stromal cell type is plated on the first surface of the porous membrane, at least one tumor cell type is present on a surface within the cell culture container; and

indirectly applying a shear stress upon the at least one tumor cell type by applying a shear stress upon a second surface of the porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

6. The method of any one of claims claim 3–5, wherein the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one tumor cell type and an upper volume comprising a second surface of the porous membrane, wherein the shear stress is applied upon the second surface of the porous membrane in the upper volume of the container.

7. The method of any one of claims 3–5, further comprising

depositing at least one extracellular matrix component on the first surface of the porous membrane and plating the at least one tumor cell type on the at least one extracellular matrix component; or

suspending the at least one tumor cell type in a solution comprising at least one extracellular matrix component to create a suspension comprising the at least one tumor cell type and the at least one extracellular matrix component, and depositing the suspension on the first surface of the porous membrane;

the porous membrane being suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one

extracellular matrix component and the at least one tumor cell type, and an upper volume comprising a second surface of the porous membrane, wherein the shear stress is applied upon the second surface of the porous membrane in the upper volume of the container.

8. The method of any one of claims 3–7, further comprising plating endothelial cells on the second surface of the porous membrane and applying the shear stress upon the plated endothelial cells, wherein the at least one tumor cell type is plated on a first surface of the porous membrane.

9. The method of any one of claims 3–7, further comprising plating at least one stromal cell type on the second surface of the porous membrane and applying the shear stress upon the plated stromal cell type.

10. The method of claim 9, further comprising plating endothelial cells on the second surface of the porous membrane.

11. The method of claim 10, comprising mixing the at least one stromal cell type with the endothelial cells prior to plating, and applying the shear stress upon the plated mixture of the at least one stromal cell type and the endothelial cells.

12. The method of claim 10, comprising sequentially plating the at least one stromal cell type and the endothelial cells on the second surface of the porous membrane.

13. The method of claim 12, comprising plating the at least one stromal cell type on the second surface of the porous membrane, subsequently plating the endothelial cells on the plated stromal cell type, and applying the shear stress on the plated endothelial cells.

14. The method of claim 12, comprising plating the endothelial cells on the second surface of the porous membrane, subsequently plating the at least one stromal cell type on the plated endothelial cells, and applying the shear stress on the plated stromal cell type.

15. The method of any one of claims 3–6, wherein the porous membrane is a first porous membrane and the method comprises plating the at least one tumor cell type on a first surface

of the first porous membrane, plating at least one stromal cell type on a second surface of the first porous membrane, placing a second porous membrane on the plated stromal cell type such that a first surface of the second porous membrane contacts the plated stromal cells, and applying the shear force upon a second surface of the second porous membrane.

16. The method of claim 7, wherein the porous membrane is a first porous membrane and the method comprises:

depositing the at least one extracellular matrix component on the first surface of the first porous membrane and plating the at least one tumor cell type on the at least one extracellular matrix component; or

depositing the suspension comprising the at least one tumor cell type and the at least one extracellular matrix component on the first surface of the first porous membrane; and

plating at least one stromal cell type on a second surface of the first porous membrane, placing a second porous membrane on the plated stromal cell type such that a first surface of the second porous membrane contacts the plated stromal cells, and applying the shear force upon a second surface of the second porous membrane.

17. The method of claim 15 or 16, further comprising plating endothelial cells on the second surface of the second porous membrane and applying the shear force upon the plated endothelial cells.

18. The method of claim 5, wherein the porous membrane is a first porous membrane and the method comprises:

plating the at least one stromal cell type on a first surface of a first porous membrane;

placing a second porous membrane on the plated stromal cell type, such that a first surface of the second porous membrane contacts the plated stromal cells;

plating at least one tumor cell type on a second surface of the second porous membrane; and

indirectly applying the shear stress upon the at least one tumor cell type by applying the shear stress upon the second surface of the first porous membrane.

19. A method for mimicking a tumor microenvironment *in vitro*, the method comprising:

adding a culture medium to a cell culture container;

plating at least one stromal cell type on a first surface of a first porous membrane within the cell culture container;

placing a second porous membrane on the plated stromal cell type, such that a first surface of the second porous membrane contacts the plated stromal cells;

plating at least one tumor cell type on a second surface of the second porous membrane; and

indirectly applying a shear stress upon the at least one tumor cell type by applying a shear stress upon the second surface of the first porous membrane, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the tumor cells are indirectly exposed *in vivo* in the tumor microenvironment.

20. The method of claim 18 or 19, wherein the first porous membrane is suspended in the cell culture container such that the first surface of the first porous membrane is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one tumor cell type, the second porous membrane, and the at least one stromal cell type, and an upper volume comprising a second surface of the first porous membrane, wherein the shear stress is applied upon the second surface of the first porous membrane in the upper volume of the container.

21. The method of any one of claims 18–20, further comprising:

depositing at least one extracellular matrix component on the second surface of the second porous membrane and plating the at least one tumor cell type on the at least one extracellular matrix component; or

suspending the at least one tumor cell type in a solution comprising at least one extracellular matrix component to create a suspension comprising the at least one tumor cell type and the at least one extracellular matrix component, and depositing the suspension on the second surface of the second porous membrane.

22. The method of any one of claims 18–21, further comprising plating endothelial cells on the second surface of the first porous membrane and applying the shear stress upon the plated endothelial cells.

23. The method of any one of claims 15–22, wherein the method further comprises immersing the second porous membrane in a solution comprising at least one extracellular matrix component prior to placing the second porous membrane on the plated stromal cell type.

24. The method of any one of claims 8, 10–14, 17, 22, and 23, wherein the method further comprises coplating at least one tumor cell type with the endothelial cells.

25. The method of claim 24, wherein the coplating comprises mixing the at least one tumor cell type with the endothelial cells prior to plating.

26. The method of claim 26, wherein the coplating comprises sequentially plating the at least one tumor cell type and the endothelial cells.

27. The method of claim 26, wherein the coplating comprises plating the at least one tumor cell type and subsequently plating the endothelial cells.

28. The method of claim 26, wherein the coplating comprises plating the endothelial cells and subsequently plating the at least one tumor cell type.

29. The method of any one of claims 24–28, wherein the coplating comprises plating the endothelial cells and the at least one tumor cell type at a ratio of about 100:1 to about 3:1.

30. The method of claim 29, wherein the coplating comprises plating the endothelial cells and the at least one tumor cell type at a ratio of about 50:1 to about 10:1.

31. The method of any one of claims 24–30, wherein the at least one tumor cell type comprises cells derived from a glioblastoma.

32. An *in vitro* method of testing a drug or a compound for an effect on a tumor, the method comprising:

mimicking the tumor microenvironment according to the method of any one of claims 1–31;

adding a drug or a compound to the culture medium; and

indirectly applying the shear stress upon the at least one tumor cell type directly or indirectly exposed to the drug or the compound; wherein a change in the at least one tumor cell type, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on the tumor.

33. The method of any one of claims 1–32, wherein upon application of the shear stress, a change in the level or localization of a marker of the tumor microenvironment in the at least one tumor cell type, the at least one stromal cell type, or in the endothelial cells, or a change in the level of a marker of the tumor microenvironment in the culture medium, as compared to the level or localization of the marker in the at least one tumor cell type, the at least one stromal cell type, or in the endothelial cells or the level of the marker in the culture medium in the absence of application of the shear stress confirms mimicking of the tumor microenvironment.

34. The method of any one of claims 1–33, further comprising inlets and outlets within the cell culture container.

35. The method of any one of claims 1–34 further comprising perfusing culture medium into and out of the cell culture container.

36. The method of any one of claims 6–17 and 20–35, wherein the cell culture container further comprises inlets and outlets within the portions of the cell culture container defining the upper and lower volumes.

37. The method of any one of claims 6–17 and 20–36, further comprising perfusing culture medium into and out of the upper volume.

38. The method of any one of claims 6–17 and 20–37, further comprising perfusing culture medium into and out of the lower volume.

39. The method of any one of claims 3–38, wherein the porous membrane, the first porous membrane, or the second porous membrane is adapted to permit fluid communication

of the culture medium and physical interaction and communication between cells plated on opposing sides of the porous membrane.

40. The method of any one of claims 1–14 and 24–39, further comprising plating at least one stromal cell type on the surface within the cell culture container, on the at least one extracellular matrix component, or on the first surface of the porous membrane; or suspending at least one stromal cell type with the tumor cell type in the solution comprising the at least one extracellular matrix component to create a suspension comprising the at least one stromal cell type, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the surface within the cell culture container or on the first surface of the porous membrane.

41. The method of any one of claims 15–17 and 24–39, further comprising plating at least one stromal cell type on the first surface of the first porous membrane; or suspending at least one stromal cell type with the tumor cell type in the solution comprising the at least one extracellular matrix component to create a suspension comprising the at least one stromal cell type, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the first surface of the first porous membrane.

42. The method of any one of claims 18–39, further comprising plating at least one stromal cell type on the second surface of the second porous membrane; or suspending at least one stromal cell type with the tumor cell type in the solution comprising the at least one extracellular matrix component to create a suspension comprising the at least one stromal cell type, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the second surface of the second porous membrane.

43. The method of any one of claims 1–42, wherein the at least one tumor cell type, the endothelial cells, or the at least one stromal cell type comprises immortalized cells.

44. The method of any one of claims 1–43, wherein the at least one tumor cell type, the endothelial cells, or the at least one stromal cell type comprises primary cells.

45. The method of any one of claims 1–44, wherein the cells comprise cells derived from an animal.

46. The method of claim 45, wherein the animal is a genetically modified animal.

47. The method of claim 45, wherein the animal is a human.

48. The method of any one of claims 1–47, wherein the at least one tumor cell type comprises cells derived from a carcinoma, a sarcoma, a lymphoma, an adenosquamous carcinoma, a mixed mesodermal tumor, carcinosarcoma, a teratocarcinoma, or a combination thereof.

49. The method of claim 48, wherein the cells derived from a carcinoma comprise cells derived from an adenocarcinoma, cells derived from a squamous cell carcinoma, or a combination thereof; wherein the cells derived from a sarcoma comprise cells derived from an osteosarcoma, a chondrosarcoma, a leiomyosarcoma, a rhabdomyosarcoma, a mesothelial sarcoma (mesothelioma), a fibrosarcoma, an angiosarcoma, a liposarcoma, a glioma, an astrocytoma, a myxosarcoma, a mesenchymous tumor, a mixed mesodermal tumor, or a combination thereof; or wherein the cells derived from a lymphoma comprise cells derived from a Hodgkin lymphoma, a non-Hodgkin lymphoma, or a combination thereof.

50. The method of claim 49, wherein the cells derived from an angiosarcoma comprise cells derived from a hemangioendothelioma, a lymphangiosarcoma, or a combination thereof.

51. The method of any one of claims 1–50, wherein the at least one tumor cell type is derived from a tumor of connective tissue, a tumor of endothelium or mesothelium, a tumor of lymphoid tissue, a tumor of muscle, a tumor of an epithelial tissue, a tumor of a neural tissue, a tumor of the amine precursor uptake and decarboxylation (APUD) system, a tumor of a neural crest-derived cell, a gonadal tumor, or a combination thereof.

52. The method of claim 51, wherein the tumor of the connective tissue comprises a tumor of adult fibrous tissue, embryonic (myxomatous) fibrous tissue, adipose tissue, cartilage tissue, bone, notochord, a fibrous histiocytoma, or a combination thereof;

wherein the tumor of endothelium or mesothelium comprises a blood vessel tumor, a lymph vessel tumor, a mesothelium tumor, or a combination thereof; wherein the tumor of lymphoid tissue comprises a plasmacytoma, a Hodgkin lymphoma, a non-Hodgkin lymphoma, or a combination thereof; wherein the tumor of muscle comprises a smooth muscle tumor or a striated muscle tumor, or a combination thereof; wherein the tumor of an epithelial tissue comprises a tumor of a stratified squamous tissue, a tumor of a glandular epithelium, a tumor of transitional epithelium, a placental tumor, a testicular tumor, or a combination thereof; wherein the tumor of a neural tissue comprises a glial cell tumor, a nerve cell tumor, a tumor of the meninges, a nerve sheath tumor, or a combination thereof; wherein the tumor of the amine precursor uptake and decarboxylation (APUD) system comprises a pituitary tumor, a parathyroid tumor, a thyroid tumor, a bronchial lining tumor, an adrenal medulla tumor, a pancreatic tumor, a tumor of the stomach or intestines, a tumor of the carotid body tumor or chemoreceptor system, or a combination thereof; wherein the tumor of a neural crest-derived cell comprises a tumor of a pigment producing cell, a tumor of a Schwann cell of the peripheral nervous system, a tumor of a Merkel cell, or a combination thereof; or wherein the gonadal tumor comprises a tumor of the ovary, a tumor of the testis, a seminoma, a dysgerminoma, a choriocarcinoma, an embryonal carcinoma, an endodermal sinus tumor, a teratocarcinoma, a Sertoli-Leydig cell tumor, an arrhenoblastoma, a granulosa-theca cell tumor, a hilar cell tumor, a lipid cell tumor, or a combination thereof.

53. The method of claim 52, wherein the tumor of adult fibrous tissue comprises a firboma or a fibrosarcoma; wherein the tumor of the embryonic (myxomatous) fibrous tissue comprises a myxoma or a myxosarcoma; wherein the tumor of the adipose tissue comprises a lipoma or a liposarcoma; wherein the tumor of the cartilage tissue comprises a chondroma or a chondrosarcoma; wherein the tumor of the bone comprises a osteoma or a osteosarcoma; wherein the tumor of the notochord comprises a chordoma; or wherein the fibrous histiocytoma comprises a malignant fibrous histiocytoma.

54. The method of claim 52, wherein the blood vessel tumor comprises a hemangioma, a hemangiopericytoma, a hemangiosarcoma, or an angiosarcoma; the lymph vessel tumor comprises a lymphangioma or a lymphangiosarcoma; or the mesothelium tumor comprises a mesothelioma.

55. The method of claim 52, wherein the smooth muscle tumor comprises a leiomyoma or a leiomyosarcoma; or the striated muscle tumor comprises a rhabdomyoma or a rhabdomyosarcoma.

56. The method of claim 52, wherein the tumor of a stratified squamous tissue comprises a papilloma, a seborrheic keratosis, a skin adnexal tumor, a squamous cell carcinoma, or an epidermoid carcinoma; wherein the tumor of the glandular epithelium comprises a tumor of the glandular epithelium or a liver, kidney or bile duct; the tumor of the transitional epithelium comprises a transitional cell papilloma or a transitional cell carcinoma; the placental tumor comprises a hydatidiform mole or a choriocarcinoma; or the testicular tumor comprises a seminoma or an embryonal cell carcinoma.

57. The method of claim 56, wherein the tumor of the glandular epithelium of a liver comprises a hepatic adenoma or a hepatocellular carcinoma; wherein the tumor of the glandular epithelium of a kidney comprises a renal tubular adenoma, a renal cell carcinoma, or a hypernephroma; or wherein the tumor of the glandular epithelium of a bile duct comprises a bile duct adenoma or a cholangiocarcinoma.

58. The method of claim 52, wherein the glial cell tumor comprises a glioma or a glioblastoma; wherein the nerve cell tumor comprises a ganglioneuroma, a nueroblastoma, or a medulloblastoma; wherein the tumor of the meninges comprises a meningioma; or wherein the nerve sheath tumor comprises a Schwannoma, a neurilemmoma, a neurofibroma, a minigioma, or a neurofibrosarcoma.

59. The method of claim 52, wherein the pituitary tumor comprises a basophilic adenoma, a eosinophilic adenoma, or a chromophobe adenoma; wherein the parathyroid tumor comprises a parathyroid adenoma or a parathyroid carcinoma; wherein the thyroid tumor comprises a C cell hyperplasia or a medullary carcinoma of the thyroid; wherein the bronchial lining tumor comprises a bronchial carcinoid or an oat cell carcinoma; wherein the adrenal medulla tumor comprises a pheochromocytoma; wherein the pancreatic tumor comprises an islet celladenoma, an insulinoma, a gastrinoma, or an islet cell carcinoma; wherein the tumor of the stomach or intestines comprises a carcinoid; or wherein the tumor of

the carotid body tumor or chemoreceptor system comprises a chemodectoma, a paraganglioma, or a carcinoid.

60. The method of claim 52, wherein the tumor of a pigment producing cell comprises a nevus or a melanoma; wherein the tumor of a Schwann cell of the peripheral nervous system comprises a Schwannoma or a neurilemmoma; or wherein the tumor of a Merkel cell comprises a Merkel cell neoplasm.

61. The method of any one of claims 1–60, wherein the at least one tumor cell type comprises cells derived from a tumor of the lung, breast, colon, rectum, prostate, bladder, bone, pancreas, liver, bile duct, ovary, testis, uterus, placenta, brain, cartilage, smooth muscle, striated muscle, membranous lining of a body cavity, fibrous tissue, blood vessel, lymph vessel, lymph node, adipose tissue, neurogenic connective tissue of the brain, kidney, pituitary gland, parathyroid, thyroid, bronchial lining, adrenalmedulla, stomach, large intestine, small intestine, carotid body, chemoreceptor system, skin, gall bladder, or a combination thereof.

62. The method of any one of claims 1–61, wherein the at least one tumor cell type comprises an immortalized cell line comprising non-small cell lung adenocarcinoma cells, breast carcinoma cells, pancreas carcinoma cells, prostate cancer cells, ovarian carcinoma cells, colon cancer cells, or a combination thereof.

63. The method of claim 62, wherein the immortalized cell line comprises human non-small cell lung adenocarcinoma cell line A549, human breast carcinoma cell line MDA-MB-231, human pancreas carcinoma cell line BxPC-3, human prostate cancer cell line DU145, human prostate cancer cell line LNCaP, human ovarian carcinoma cell line SKOV-3, human colon cancer cell line COLO-205, or a combination thereof.

64. The method of any one of claims 1–61, wherein the at least one tumor cell type comprises primary tumor cells obtained from a subject by biopsy, tumor resection, blood draw, or a combination thereof.

65. The method of claim 64, wherein the primary tumor cells are obtained from a stage I tumor, a stage II tumor, a stage III tumor, or a stage IV tumor.

66. The method of any one of claims 1–61, 64, and 65, wherein the at least one tumor cell type comprises tumor cells derived from a humanized mouse bearing a tumor derived from a human subject.

67. The method of claim 66, wherein the humanized mouse comprises a non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse, a NOD/Shi-scid/IL-2Rγnull (NOG) mouse, or a NOD SCID IL-2Rγ knockout (NSG) mouse.

68. The method of any one of claims 8, 10–14. 17, and 22–67, wherein the endothelial cells comprise microvascular endothelial cells, macrovascular endothelial cells, endothelial progenitor cells, or a combination thereof.

69. The method of any one of clams 8, 10–14. 17, and 22–68, wherein the endothelial cells comprise endothelial cells derived from a tumor, or wherein the endothelial cells comprise endothelial cells derived from an organ or tissue in which a tumor resides.

70. The method of any one of claims 8, 10–14. 17, and 22–69, wherein the endothelial cells comprise endothelial cells derived from lung, breast, colon, rectum, prostate, bladder, bone, pancreas, liver, bile duct, ovary, testis, uterus, placenta, brain, cartilage, smooth muscle, striated muscle, a membranous lining of a body cavity, fibrous tissue, blood vessel, lymph vessel, lymph node, adipose tissue, neurogenic connective tissue of the brain, kidney, pituitary gland, parathyroid, thyroid, bronchial lining, adrenalmedulla, stomach, large intestine, small intestine, carotid body, chemoreceptor system, skin, gall bladder, or a combination thereof.

71. The method of any one of claims 68–70, wherein the endothelial cells comprise the microvascular endothelial cells, the microvascular endothelial cells comprising lung microvascular endothelial cells, breast microvascular endothelial cells, pancreatic microvascular endothelial cells, prostate microvascular endothelial cells, ovarian

microvascular endothelial cells, colon microvascular endothelial cells, dermal microvascular endothelial cells, or a combination thereof.

72. The method of any one of claims 8, 10–14. 17, and 22–71, wherein the endothelial cells comprise cells derived from inducible pluripotent stem cells (iPSC).

73. The method of any one of claims 9–72, wherein the at least one stromal cell type comprises fibroblasts, immune cells, pericytes, inflammatory cells, or a combination thereof.

74. The method claim 73, wherein the at least one stromal cell type comprises the immune cells, the immune cells comprising macrophages, lymphocytes, dendritic cells, or a combination thereof.

75. The method of claim 73 or 74, wherein the at least one stromal cell type comprises the inflammatory cells, the inflammatory cells comprising B cells, T cells, or a combination thereof.

76. The method of any one of claims 9–75, wherein the at least one stromal cell type comprises cells derived from inducible pluripotent stem cells (iPSC).

77. The method of any one of claims 40–76, further comprising mixing the at least one stromal cell type with the at least one tumor cell type prior to plating.

78. The method of claim 77, wherein the at least one stromal cell type is mixed with the at least one tumor cell type at a ratio of about 0.1:1 to about 3:1.

79. The method of claim 78, wherein the at least one stromal cell type is mixed with the at least one tumor cell type at a ratio of about 0.2:1 to about 2:1.

80. The method of claim 78, wherein the at least one stromal cell type is mixed with the at least one tumor cell type at a ratio of about 0.25:1.

81. The method of claim 78, wherein the at least one stromal cell type is mixed with the at least one tumor cell type at a ratio of about 1:1.

82. The method of any one of claims 40–76, wherein the method comprises sequentially plating the at least one tumor cell type and the at least one stromal cell type.

83. The method of claim 82, comprising plating the at least one tumor cell type and subsequently plating the at least one stromal cell type on the plated tumor cell type.

84. The method of claim 82, comprising plating the at least one stromal cell type and subsequently plating the at least one tumor cell type on the plated stromal cell type.

85. The method of any one of claims 1–84, further comprising plating one or more additional cell types on a surface of the cell culture container, on the at least one extracellular matrix component, on the first or second surface of the porous membrane, on the first or second surface of the first porous membrane, or on the first or second surface of the second porous membrane; or suspending one or more additional cell types in the culture medium within the upper volume or in the culture medium within the lower volume.

86. The method of any one of claims 2, 7–14, 16, 17, and 21–85, further comprising suspending one or more additional cell types with the at least one tumor cell type in the solution comprising the at least on extracellular matrix component to create a suspension comprising the one or more additional cell types, the at least one tumor cell type, and the at least one extracellular matrix component, and depositing the suspension on the surface within the cell culture container, on the first surface of the porous membrane, on the first surface of the first porous membrane, or on the second surface of the second porous membrane.

87. The method of claim 85 or 86, wherein the one or more additional cell types comprises primary cells.

88. The method of any one of claims 85–87, wherein the one or more additional cell types comprises immortalized cells.

89. The method of any one of claims 85–88, wherein the one or more additional cell types comprises an animal cell type.

90. The method of claim 89, wherein the animal cell type is a human cell type.

91. The method of any one of claims 85–90, wherein the one or more additional cell types comprise a cell type adhered to the bottom surface of the cell culture container.

92. The method of any one of claims 85–91, wherein the one or more additional cell types comprise fibroblasts, immune cells, pericytes, inflammatory cells, or a combination thereof.

93. The method of claim 73 or 92, wherein the at least one stromal cell type or the one or more additional cell types comprises the fibroblasts, the fibroblasts comprising fetal stromal fibroblasts.

94. The method of claim 93, wherein the fetal stromal fibroblasts comprise human fetal stromal fibroblast cell line IMR-90.

95. The method of claim 73 or 92, wherein at least one stromal cell type or the one or more additional cell types comprises the fibroblasts, the fibroblasts comprising human lung fibroblast cell line Hs888Lu.

96. The method of any one of claims 92–95, wherein the one or more additional cell types comprises the immune cells, the immune cells comprising macrophages, lymphocytes, dendritic cells, or a combination thereof.

97. The method of claim 96, wherein the immune cells comprise the lymphocytes and the lymphocytes are suspended in the culture medium within the upper volume.

98. The method of any one of claims 92–97, wherein the one or more additional cell types comprises the inflammatory cells, the inflammatory cells comprising B cells, T cells, or a combination thereof.

99. The method of any one of claims 1–98, further comprising the step of culturing the cell type or cell types.

100. The method of any one of claims 1, 3–6, 8–15, 17–20, and 22–100, wherein the method comprises culturing the cell type or cell types in the substantial absence of exogenously added extracellular matrix.

101. The method of any one of claims 2, 7–14, 16, 17, and 21–99, wherein the at least one extracellular matrix component comprises a collagen, heparan sulfate, chondroitin sulfate, keratan sulfate, hyaluronic acid, an elastin, a fibronectin, a laminin, a vitronectin, or a combination thereof.

102. The method of claim 101, wherein the at least one extracellular matrix component comprises the collagen.

103. The method of claim 102, wherein the collagen comprises collagen type I, collagen type II, collagen type III, collagen type IV, collagen type V, collagen type VI, collagen type VII, collagen type VIII, collagen type IX, collagen type X, collagen type XI, collagen type XII, collagen type XIII, collagen type XIV, collagen type XV, collagen type XVI, collagen type XVII, collagen type XVIII, collagen type XIX, collagen type XX, collagen type XXI, collagen type XXII, collagen type XXIII, collagen type XXIV, collagen type XXV, collagen type XXVI, collagen type XXVII, collagen type XXVIII, or a combination thereof.

104. The method of claim 103, wherein the collagen comprises the collagen type I.

105. The method of any one of claims 102–104, wherein the concentration of the collagen is about 1 mg/ml to about 10 mg/ml.

106. The method of claim 105, wherein the concentration of the collagen is about 2 mg/ml to about 5 mg/ml.

107. The method of claim 105, wherein the concentration of the collagen is at least about 2 mg/ml.

108. The method of any one of claims 2, 7–14, 16, 17, and 21–107, wherein the at least one extracellular matrix component comprises decellularized extracellular matrix purified from a biological source.

109. The method of claim 108, wherein the biological source comprises human placenta.

110. The method of any one of claims 1–109, wherein the cell type or cell types secrete at least one extracellular matrix component.

111. The method of claim 110, wherein the at least one tumor cell type secretes at least one extracellular matrix component.

112. The method of any one of claims 1–111, wherein the method further comprises plating fibroblasts, chondrocytes, or osteoblasts on a surface within the cell culture container, wherein the fibroblasts, chondrocytes, or osteoblasts secrete at least one extracellular matrix component.

113. The method of any one of claims 2, 7–14, 16, 17, and 21–112, wherein the at least one extracellular matrix component has a Young's modulus of about 0.1 kPa to about 25 kPa.

114. The method of claim 113, wherein the at least one extracellular matrix component has a Young's modulus of about 0.15 kPa to about 15 kPa.

115. The method of claim 113, wherein the at least one extracellular matrix component has a Young's modulus of about 3 kPa to about 12 kPa.

116. The method of any one of claims 2, 7–14, 16, 17, and 21–115, wherein the Young's modulus of the at least one extracellular matrix component is non-uniform.

117. The method of any one of claims 2, 7–14, 16, 17, and 21–116, wherein the method further comprises depositing an additional layer of at least one extracellular matrix

component on top of the at least one tumor cell type, such that the at least one extracellular matrix component substantially surrounds the at least one tumor cell type.

118. The method of claim 117, wherein the additional layer of at least one extracellular matrix component has a Young's modulus that is different from the Young's modulus of the at least one extracellular matrix component deposited on the surface within the cell culture container, on the first surface of the porous membrane, on the first surface of the first porous membrane, or on the second surface of the second porous membrane.

119. The method of claim 117 or 118, wherein the Young's modulus of the additional layer of at least one extracellular matrix component is non-uniform.

120. The method of any one of claims 1–119, wherein the culture medium comprises GM-CSF, TGF-β, or a combination thereof.

121. The method of any one of claims 1–120, wherein the culture medium comprises sera, blood, blood cells, a blood component, immune cells, conditioned culture medium, or a combination thereof.

122. The method of claim 121, wherein the sera, blood, blood cells, blood component, or immune cells are derived from a human or an animal.

123. The method of claim 122, wherein the animal comprises a mouse, rat, guinea pig, hamster, rabbit, cat, dog, monkey, cow, pig, horse, goat, sheep, bird, or fish.

124. The method of any one of claims 121–123, wherein the immune cells comprise B cells, dendritic cells, granulocytes, innate lymphoid cells, megakaryocytes, monocytes, macrophages, natural killer cells, T cells, thymocytes, or a combination thereof.

125. The method of any one of claims 121–124, wherein the blood cells comprise platelets, red blood cells, or a combination thereof.

126. The method of any one of claims 121–125, wherein the blood component comprises a clotting factor, a lipoprotein, a triglyceride, or a combination thereof.

127. The method of any one of claims 121–126, wherein the conditioned culture medium comprises conditioned culture medium from a culture comprising tumor cells, a culture comprising endothelial cells, a culture comprising a stromal cell type, or a combination thereof.

128. The method of any one of claims 1–127, wherein the method comprises mimicking the tumor microenvironment *in vitro* in a first cell culture container according to the method of any one of claims 1–127, mimicking the tumor microenvironment *in vitro* in a second cell culture container according to the method of any one of claim 1–127, and transferring cells of the at least one tumor cell type cultured in the first cell culture container into the second cell culture container.

129. The method of claim 128, wherein the transferring comprises manually transferring the cells of the at least one tumor cell type cultured in the first cell culture container into the second cell culture container.

130. The method of claim 128, wherein an outlet in the first cell culture container is connected to an inlet in the second cell culture container, and the method further comprises pumping culture medium comprising the at least one tumor cell type out of the first cell culture container and into the second cell culture container.

131. The method of any one of claims 1–130, wherein the method further comprises introducing cells cultured in an *in vitro* system that models an organ or tissue into the cell culture container.

132. A method for mimicking tumor metastasis *in vitro*, comprising introducing cells of the at least one tumor cell type cultured according to the method of any one of claims 1–131 into an *in vitro* system that models an organ or tissue.

133. The method of claim 131 or 132, wherein the *in vitro* system that models the organ or tissue models liver, pancreas, bone, lung, blood vessels, the lymphatic system, brain, muscle, bladder, kidney, intestine, colon, gall bladder, skin, or bone.

134. The method of claim 133, wherein the *in vitro* system that models the organ or tissue models the liver.

135. The method of claim 134, wherein the *in vitro* system that models the liver comprises another cell culture container comprising a culture medium and a porous membrane, wherein hepatocytes are plated on a first surface of the porous membrane and the porous membrane is suspended in the another cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the container, thereby defining within the container a lower volume comprising the hepatocytes and an upper volume comprising a second surface of the porous membrane; wherein a shear stress is applied upon the second surface of the porous membrane in the upper volume, the shear stress mimicking flow to which the hepatocytes are exposed *in vivo*; and wherein the another cell culture container further comprises inlets within the portions of the cell culture container defining the upper and lower volumes.

136. The method of claim 135, wherein introducing the cells of the at least one tumor cell type into the *in vitro* system that models the liver comprises transferring the cells of the at least one tumor cell type into the lower volume of the *in vitro* system that models the liver.

137. The method of claim 135, wherein introducing the cells of the at least one tumor cell type into the *in vitro* system that models the liver comprises transferring the cells of the at least one tumor cell type into the upper volume of the *in vitro* system that models the liver.

138. The method of claim 137, further comprising assessing migration of the cells of the at least one tumor cell type into the lower volume of the *in vitro* system.

139. The method of any one of claims 136–138, wherein the transferring comprises manually transferring the cells of the at least one tumor cell type into the lower volume or the upper volume of the *in vitro* system that models the liver.

140. The method of any one of claims 136–138, wherein the cell culture container comprising the at least one tumor cell type further comprises an outlet within the portion of the cell culture container defining the lower volume and containing the at least one tumor cell type, said outlet is connected to an inlet in the another cell culture container of the *in vitro* system that models the liver, and the transferring comprises pumping the culture medium out of the lower volume of the cell culture container comprising the at least one tumor cell type and into the upper or lower volume of the another cell culture container of the *in vitro* system that models the liver.

141. The method of any one of claims 136–138, wherein the cell culture container comprising the at least one tumor cell type further comprises an outlet within the portion of the cell culture container defining the upper volume, said outlet being connected to an inlet in the another cell culture container of the *in vitro* system that models the liver, and the transferring comprises pumping the culture medium out of the upper volume of the cell culture container and into the upper or lower volume of the another cell culture container of the *in vitro* system that models the liver.

142. A method for mimicking tumor metastasis, comprising introducing cells of the at least one tumor cell type cultured according to the method of any one of claims 1–131 into an animal.

143. The method of claim 142, wherein the animal is a mammal.

144. The method of claim 143, wherein the mammal is a mouse, rat, guinea pig, hamster rabbit, cat, dog, monkey, cow, pig, horse, goat, or sheep.

145. The method of claim 144, wherein the animal is a bird or fish.

146. The method of claim 144, wherein the mouse is a humanized mouse and the at least one tumor cell type comprises a human tumor cell type.

147. The method of claim 146, wherein the humanized mouse comprises a non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse, a NOD/Shi-scid/IL-2Rγnull (NOG) mouse, or a NOD SCID IL-2Rγ knockout (NSG) mouse.

148. A method for mimicking tumor metastasis *in vitro* comprising:

adding a culture medium to a cell culture container;

plating at least one cell type on a first surface of a porous membrane within the cell culture container, wherein the porous membrane is suspended in the cell culture container such that the first surface is proximal and in spaced relation to a bottom surface of the cell culture container, thereby defining within the cell culture container a lower volume comprising the at least one cell type and an upper volume comprising a second surface of the porous membrane;

indirectly applying a shear stress upon the at least one cell type, the shear stress resulting from flow of the culture medium induced by a flow device, the flow mimicking flow to which the cells are indirectly exposed *in vivo*; and

introducing tumor cells derived from a human or a humanized animal into the upper volume or the lower volume.

149. The method of claim 148, wherein the at least one cell type comprises hepatocytes or smooth muscle cells.

150. The method of claim 148 or 149, wherein the method further comprises plating a second cell type on the second surface of the porous membrane.

151. The method of claim 150, wherein the second cell type comprises endothelial cells.

152. The method of any one of claims 148–151, wherein the humanized animal is a humanized mouse.

153. The method of claim 152, wherein the humanized mouse comprises a non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse, a NOD/Shi-scid/IL-2Rγnull (NOG) mouse, or a NOD SCID IL-2Rγ knockout (NSG) mouse.

154. An *in vitro* method of testing a drug or a compound for an effect on tumor metastasis, the method comprising:

mimicking tumor metastasis *in vitro* according to the method of any one of claims 132–141 and 148–153;

adding a drug or a compound to the culture medium;

wherein a change in the cells of the at least one tumor cell type in the *in vitro* system that models the organ or tissue, in the presence of the drug or the compound, indicates that the drug or the compound has an effect on tumor metastasis.

155. A method for selecting a chemotherapy regimen to be administered to a subject having a tumor, the method comprising:

testing a drug or a compound *in vitro* for an effect on the tumor according to any one of claims 32–131 or testing a drug or a compound for an *in vitro* effect on tumor metastasis according to claim 154, wherein the at least one tumor cell type comprises tumor cells derived from the subject's tumor; and

determining whether to administer the drug or the compound to the subject based on the results of the *in vitro* testing.

156. The method of claim 155, further comprising selecting a dose of the drug or the compound to be administered to the subject based on the results of the *in vitro* testing.

157. The method of claim 155 or 156, further comprising selecting a rate of administration of the drug or the compound to be administered to the subject based on the results of the *in vitro* testing.

158. The method of any one of claims 32–147 and 154–157 wherein the concentration of the drug or compound in the culture medium is within the concentration range of the drug or the compound that achieves the effect *in vivo*.

159. The method of any one of claims 32–147 and 154–157, wherein the concentration of the drug or the compound in the culture medium is within the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound.

160. The method of claim 159, wherein the concentration of the drug or the compound in the culture medium is approximately the same as the *in vivo* therapeutic C_{max} for the drug or the compound.

161. The method of any one of claims 32–147 and 154–157, wherein the concentration of the drug or the compound in the culture medium is about 2-fold to about 20-fold lower than the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound.

162. The method of claim 161, wherein the concentration of the drug or the compound in the culture medium is about 5-fold to about 15-fold lower than the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound.

163. The method of claim 161, wherein the concentration of the drug or the compound in the culture medium is about 10-fold lower than the concentration range of the *in vivo* therapeutic C_{max} for the drug or the compound.

164. The method of any one of claims 32–147 and 154–163, wherein the effect comprises a toxic effect, a protective effect, a pathologic effect, a disease-promoting effect, an inflammatory effect, an oxidative effect, an endoplasmic reticulum stress effect, a mitochondrial stress effect, an apoptotic effect, a necrotic effect, an autophagic effect, an immunogenic cell death effect, a ferroptotic effect, a remodeling effect, a proliferative effect, an effect on angiogenesis, an effect on the activity of a protein, or an effect on the expression of a gene.

165. The method of claim 164, wherein the effect comprises the effect on the activity of a protein, the effect comprising inhibition of the protein or activation of the protein.

166. The method of claim 164, wherein the effect comprises the effect on the expression of a gene, the effect comprising an increase in the expression of the gene or a decrease in the expression of the gene.

167. The method of any one of claims 32–147 and 154–166, wherein the drug comprises an anti-cancer agent.

168. The method of claim 167, wherein the anti-cancer agent comprises an alkylating agent, an anti-metabolite, an anti-tumor antibiotic, a topoisomerase inhibitor, a corticosteroid, an anti-microtubule agent, a kinase inhibitor, a pathway inhibitor, a differentiating agent, a hormone therapy, an immunotherapy, L-asparaginase, a chelating agent, an ATP mimetic, a biologic medical product, or a combination thereof.

169. The method of claim 168, wherein the anti-cancer agent comprises the alkylating agent, the alkylating agent comprising altretamine, bendamustine, busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, ifosfamide, lomustine, mechlorethamine, melphalan, oxalaplatin, palifosamide, streptozocin, temozolomide, thiotepa, or a combination thereof; wherein the anti-cancer agent comprises the anti-metabolite, the antimetabolite comprising azathioprine, capecitabine, cladribine, clofarabine, cytarabine, floxuridine, fludarabine, fluorouracil, gemcitabine, hydroxyurea, mercaptopurine, methotrexate, nelarabine, pemetrexed, pentostatin, pralatrexate, raltitrexed, thioguanine, or a combination thereof; wherein the anti-cancer agent comprises the anti-tumor antibiotic, the anti-tumor antibiotic comprising bleomycin, dactinomycin, mitomycin, plicamycin, rifampicin, or a combination thereof; wherein the anti-cancer agent comprises the topoisomerase inhibitor, the topoisomerase inhibitor comprising amsacrine, topotecan, irinotecan , etoposide, teniposide, mitoxantrone, etirinotecan, camptothecin, daunorubicin, doxorubicin, epirubicin, idarubicin, valrubicin, amonafide, or a combination thereof; wherein the anti-cancer agent comprises the corticosteroid, the corticosteroid comprising prednisone, methylprednisolone, dexamethasone, cortisol sodium succinate, or a combination thereof; wherein the anti-cancer agent comprises the anti-microtubule agent, the anti-microtubule agent comprising vinblastine, vincristine, vindesine, vinorelbine, paclitaxel, docetaxel, ixabepilone, eribulin mesylate, cabazitaxel, or a combination thereof; wherein the anti-cancer agent comprises the kinase inhibitor, the kinase inhibitor comprising a small molecule inhibitor of a receptor or non-receptor tyrosine kinase, a serine/threonine-specific kinase inhibitor, a dual-specificity kinase inhibitor, or a combination thereof; wherein the anti-cancer agent comprises the pathway inhibitor, the pathway inhibitor comprising a B-cell lymphoma 2 (Bcl-2) family inhibitor, a heat shock protein 90 (HSP-90) inhibitor, a proteasome inhibitor, a cyclin-dependent kinase inhibitor, an inhibitor of poly ADP-ribose polymerase (PARP), an inhibitor of the mammalian target of rapamycin (mTOR), an inhibitor

of histone deacetylase (HDAC), an inhibitor of the hedgehog pathway, a rho kinase inhibitor, or a combination thereof; wherein the anti-cancer agent comprises the differentiating agent, the differentiating agent comprising a retinoid, tretinoin, bexarotene, arsenic trioxide, or a combination thereof; wherein the anti-cancer agent comprises the hormone therapy, the hormone therapy comprising a selective androgen-receptor modulator (SARM), an androgen receptor antagonist, a selective estrogen receptor modulator (SERM), an estrogen receptor antagonist, a progestin, an estrogen, an aromatase inhibitor, a gonadotropin-releasing hormone (GnRH) agonist or analog, ketoconazole, abiraterone, or a combination thereof; wherein the anti-cancer agent comprises the immunotherapy, the immunotherapy comprising a monoclonal antibody, a non-specific immunotherapy or adjuvant, an immunomodulating drug, a cancer vaccine, a targeted immunotherapy, or a combination thereof; or wherein the anti-cancer agent comprises the chelating agent, the chelating agent comprising penicillamine, triethylene tetramine dihydrochloride, EDTA, DMSA, deferoxamine mesylate, or batimastat, or a combination thereof.

170. The method of claim 169, wherein the kinase inhibitor comprises an epidermal growth factor (EGF) receptor inhibitor, a fibroblast growth factor (FGF) receptor inhibitor, a platelet-derived growth factor (PDGF) receptor inhibitor, a vascular endothelial growth factor (VEGF) receptor inhibitor, or a rho kinase inhibitor.

171. The method of claim 169, wherein the kinase inhibitor comprises the small molecule inhibitor of a receptor or non-receptor tyrosine kinase, the small molecule inhibitor of a receptor or non-receptor tyrosine kinase comprising afatinib, alectinib, alisertib, amuvatinib, apatinib, axitinib, bafetinib, barasertib, baricitinib, bosutinib, brivanib, buparlisib, cabozantinib, canertinib, cenisertib, cobimetinib, crenolanib, crizotinib, dabrafenib, dacomitinib, danusertib, desatinib, dovitinib, epitinib, erlotinib, foretinib, fostamatinib, galunisertib, gefitinib, ibrutinib, imatinib, lapatinib, lenvatinib, lestaurtinib, linifanib, linsitinib, masitinib, momelotinib, motesanib, mubritinib, neratinib, nilotinib, nintedanib, orantinib, pacritinib, pazopanib, pelitinib, pimasertib, ponatinib, poziotinib, quizartinib, refametinib, regorafenib, ruxolitinib, selumetanib, sorafenib, sulfatinib, sunitinib, tandutinib, telatinib, theliatinib, tivantinib, tofacitinib, trametinib, vandetanib, vatalinib, vemurafenib, volasertib, volitinib, or a combination thereof; or wherein the kinase inhibitor comprises the

serine/threonine-specific kinase inhibitor, the serine/threonine-specific kinase inhibitor comprising MK2206.

172. The method of claim 169, wherein the pathway inhibitor comprises the Bcl-2 family inhibitor, the Bcl-2 family inhibitor comprising navitoclax, obatoclax, oblimerson, cinacalcet, or a combination thereof; wherein the pathway inhibitor comprises the HSP-90 inhibitor, the HSP-90 inhibitor comprising tanespimycin, retaspimycin, ganetespib, or a combination thereof; wherein the pathway inhibitor comprises the proteasome inhibitor, the proteasome inhibitor comprising bortezomib, carfilzomib, oprozomib, ixazomib, marozomib, delanzomib, or a combination thereof; wherein the pathway inhibitor comprises the cyclin-dependent kinase inhibitor, the cyclin-dependent kinase inhibitor comprising flavopiridol, alvocidib, dinaciclib, seliciclib, palbociclib, or a combination thereof; wherein the pathway inhibitor comprises the inhibitor of PARP, the inhibitor of PARP comprising iniparib, veliparib, olaparib, rucaparib, niraparib, or a combination thereof; wherein the pathway inhibitor comprises the inhibitor of mTOR, the inhibitor of mTOR comprising deforolimus, everolimus, sirolimus, temsirolimus, or a combination thereof; wherein the pathway inhibitor comprises the inhibitor of HDAC, the inhibitor of HDAC comprising belinostat, entinostat, mocetinostat, panobinostat, romidepsin, vorinostat, or a combination thereof; wherein the pathway inhibitor comprises the inhibitor of the hedgehog pathway, the inhibitor of the hedgehog pathway comprising varidegib, vismodegib, or a combination thereof; or wherein the pathway inhibitor comprises the rho kinase inhibitor, the rho kinase inhibitor comprising Y27632.

173. The method of claim 169, wherein the hormone therapy comprises the SARM, the SARM comprising enobosarm; wherein the hormone therapy comprises the androgen receptor antagonist, the androgen receptor antagonist comprising bicalutamide, flutamide, nilutamide, enzalutamide, or a combination thereof; wherein the hormone therapy comprises the SERM, the SERM comprising tamoxifen, toremifene, raloxifene, or a combination thereof; wherein the hormone therapy comprises the estrogen receptor antagonist, the estrogen receptor antagonist comprising fulvestrant; wherein the hormone therapy comprises the progestin, the progestin comprising megestrol acetate; wherein the hormone therapy comprises the estrogen, the estrogen comprising estramustine; wherein the hormone therapy comprises the aromatase inhibitor, the aromatase inhibitor comprising anastrozole,

exemestane, letrozole, or a combination thereof; or wherein the hormone therapy comprises the GnRH agonist or analog, the GnRH agonist or analog comprising leuprolide, goserelin, abarelix, degarelix, triptorelin, or a combination thereof.

174. The method of claim 169, wherein the immunotherapy comprises the monoclonal antibody, the monoclonal antibody comprising rituximab, alemtuzumab, bevacizumab, abagovomab, etaracizumab, or a combination thereof; wherein the immunotherapy comprises the non-specific immunotherapy or adjuvant, the non-specific immunotherapy or adjuvant comprising interleukin-2 (IL-2), interferon-α, interferon-α2b, peginterferon alfa-2b, abatacept, aldesleukin, or a combination thereof; wherein the immunotherapy comprises the immunomodulating drug, the immunomodulating drug comprising thalidomide, lenalidomide, or a combination thereof; wherein the immunotherapy comprises the cancer vaccine, the cancer vaccine comprising Sipuleucel-T, Bacillus Calmette-Guérin (BCG) vaccine, or a combination thereof; or wherein immunotherapy comprises the targeted immunotherapy, the targeted immunotherapy comprising brentuzimab, cetuximab, ibritumomab, ipilimumab, ofatumumab, panitumumab, pertuzumab, tositmuomab, trastuzumab, tremelimumab, siltuximab, tocilizumab, canakinumab, lirilumab, nivolumab, pidilizumab, lambrolizumab, or a combination thereof.

175. The method of claim 168, wherein the anti-cancer agent comprises the biologic medical product, the biologic medical product comprising a synthetic polysaccharide; a synthetic, partially synthetic or humanized immunoglobulin; a recombinant therapeutic protein; or a combination thereof.

176. The method of any one of claims 32–147 and 154–175, wherein the drug or the compound comprises a radiocontrast agent, a radio-isotope, a prodrug, an antibody fragment, an antibody, a live cell, a therapeutic drug delivery microsphere, microbead, nanoparticle, gel or cell-impregnated gel, or a combination thereof.

177. The method of any one of claims 32–147 and 154–175, wherein adding the drug or the compound to the culture medium comprises adding an antibody-drug conjugate or a modified release dosage form comprising the drug or the compound to the culture medium.

178. The method of claim 177, wherein the modified release dosage form comprises an oral modified release dosage form.

179. The method of claim 177 or 178, wherein the modified release dosage form comprises a modified release polymer.

180. The method of claim 179, wherein the modified release polymer comprises hydroxypropylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, ethylcellulose, methylcellulose, carboxymethylcellulose, alginic acid, carrageenan, chitosan, heparin, starch, xanthan gum, polyvinyl alcohol, polyacrylic acid, polyethylene oxide, poloxamers, pluronics, polymethacrylate, polysialic acid, or a combination thereof.

181. The method of any one of claims 32–147 and 154–180, wherein the drug or compound is capable of inhibiting, activating, or altering the function of proteins or genes in the at least one cell type.

182. The method of any one of claims 32–147 and 154–181, wherein the method further comprises perfusing the drug or the compound into at least one of the upper volume and the lower volume.

183. The method of claim 182, wherein the method further comprises perfusing the drug or the compound into the upper volume.

184. The method of claim 182 or 183, wherein the method further comprises perfusing the drug or the compound into the lower volume.

185 The method of any one of claims 1–184, wherein the flow is derived from a previously measured hemodynamic pattern and is modeled into a set of electronic instructions, and the shear stress is based on the set of electronic instructions.

186. The method of any one of claims 6–17 and 20–185, wherein the flow device comprises a body adapted for being positioned in the culture medium in the upper volume of the cell culture container and a motor adapted to rotate the body.

187. The method of claim 186, wherein the body has a conical surface or a flat surface.

188. The method of claim 187, wherein the flow device is adapted for positioning the conical or flat surface of the body in the cell culture container and in contact with the culture medium.

189. The method of any one of claims 185–188, wherein the flow device comprises an electronic controller for receiving the set of electronic instructions and a motor operated by the electronic controller.

190. The method of claim 189, wherein the flow device further comprises a shear stress applicator operatively connected to the motor for being driven by the motor.

191. The method of claim 190, wherein the shear stress applicator comprises a cone or a disc attached to the motor.

192. The method of any one of claims 185–191, wherein the hemodynamic pattern is derived from the vasculature of a tumor.

193. The method of claim 192, wherein the hemodynamic pattern is derived from at least a portion of a capillary, an arteriole, an artery, a venule, or a vein.

194. The method of claim 192 or 193, wherein the hemodynamic pattern is derived from at least a portion of an organ, the organ comprising a liver, a kidney, a lung, a brain, a pancreas, a spleen, a large intestine, a small intestine, a heart, a skeletal muscle, an eye, a tongue, a reproductive organ, or an umbilical cord.

195. The method of any one of claims 185–194, wherein the hemodynamic pattern is derived from analysis of ultrasound data.

196. The method of any one of claims 185–194, wherein the hemodynamic pattern is derived from analysis of magnetic resonance imaging (MRI) data.

197. The method of any one of claims 2–196, wherein the flow or the hemodynamic pattern is time-variant.

198. The method of any one of claims 1–197, wherein the shear stress applied upon the at least one tumor cell type is about 0.1 dynes/cm^2 to about 200 dynes/cm^2.

199. The method of claim 198, wherein the shear stress applied upon the at least one tumor cell type is about 0.1 dynes/cm^2 to about 100 dynes/cm^2.

200. The method of any one of claims 1–199, wherein the shear stress is applied at a rate of about 1 sec^{-1} to about 1000 sec^{-1}.

201. The method of any one of claims 1–200, wherein the flow or the hemodynamic pattern is derived from an animal.

202. The method of claim 201, wherein the animal is a genetically modified animal.

203. The method of claim 201, wherein the animal is a human.

204. The method of any one of claims 132–141 and 148–203, wherein upon application of the shear stress, a change in the level or localization of a marker of the tumor metastasis in the at least one tumor cell type, or a change in the level of a marker of the tumor metastasis in the culture medium, as compared to the level or localization of the marker in the at least one tumor cell type or the level of the marker in the culture medium in the absence of application of the shear stress confirms mimicking of the tumor metastasis.

205. The method of any one of claims 33–141 and 148–204, wherein the marker comprises a marker of cell proliferation, cell invasion, angiogenesis, tumorigenesis, cell monolayer integrity, endothelial cell barrier function, permeability, inflammation, cell death, apoptosis, necrosis, contraction, cell motility, or a combination thereof.

206. The method of claim 204 or 205, wherein the change in the level of a marker is an increase in the level of the marker in the at least one tumor cell type or the endothelial cells.

207. The method of claim 204 or 205, wherein the change in the level of a marker is a decrease in the level of the marker in the at least one tumor cell type or the endothelial cells.

208. The method of any one of claims 204–207, wherein the marker comprises VE-cadherin, E-cadherin, actin, or a combination thereof.

209. The method of any one of claims 204–208, wherein the marker comprises a marker of angiogenesis, and the marker of angiogenesis comprises vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, angiopoietin-1 (ANG1), angiopoietin-2 (ANG2), fibroblast growth factor-2 (FGF-2), placental growth factor (PLGF), or a combination thereof; wherein the marker comprises a marker of cell proliferation, and the marker of cell proliferation comprises epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), MKI67, proliferating cell nuclear antigen (PCNA), or a combination thereof; wherein the marker comprises a marker of cell invasion, and the marker of cell invasion comprises vimentin (VIM), cadherin 1 (CDH-1), cadherin 2 (CDH-2), or a combination thereof; wherein the marker comprises a marker of inflammation, and the marker of inflammation comprises interleukin-6 (IL-6), interleukin-8 (IL-8), NF-κB, endothelial nitric oxide synthase (eNOS), Krupple-like factor 2 (KLF2), monocyte chemotactic protein-1 (MCP-1), or a combination thereof; or a combination thereof.

210. The method of any one of claims 8–14, 17, and 22–209, wherein the method further comprises analyzing the endothelial cells for cell density, monolayer integrity, permeability, or a combination thereof.

211. The method of any one of claims 1–210, wherein the method further comprises analyzing the morphology of the at least one tumor cell type, the endothelial cells, the at least one stromal cell type, or the one or more additional cell types.

212. The method of any one of claims 1–211, further comprising analyzing the culture medium for cytokine secretion, chemokine secretion, humoral factor secretion, microparticle

secretion, growth factor secretion, shedding of a protein from the cellular surface, a metabolite of a compound, an immune cell, nitric oxide secretion, a vasodilator protein, a vasoconstrictive protein, miRNA, a secreted protein, or a secreted biological substance.

213. The method of claim 212, wherein the culture medium is analyzed for shedding of a protein from the cellular surface, and the protein comprises a vascular cell adhesion molecule (VCAM), E-selectin, or an intracellular adhesion molecule (ICAM).

214. The method of claim 212 or 213, wherein the culture medium is analyzed for nitric oxide secretion by measuring nitrate or nitrite concentration.

215. The method of any one of claims 32–147 and 154–214, further comprising analyzing the at least one tumor cell type, the endothelial cells, the at least one stromal cell, or the one or more additional cell types type for toxicity, inflammation, permeability, compatibility, cellular adhesion, cellular remodeling, cellular migration, or phenotypic modulation resulting from the drug or the compound.

216. The method of any one of claims 32–147 and 154–215, further comprising comparing at least one of the cell types after applying the shear stress for a period of time wherein the medium includes the drug or the compound to the at least one of the cell types after applying the shear stress for the period of time wherein the medium does not include the drug or the compound, to determine the effect of the drug or compound on the at least one of the cell types.

217. The method of any one of claims 1–216, wherein the method further comprises identifying a drug target.

218. The method of any one of claims 1–217, wherein the method further comprises identifying a surface protein of the at least one tumor cell type, the at least one stromal cell type, the endothelial cells, or the one or more additional cell types as a target for a drug delivery modality.

219. The method of claim 218, wherein the drug delivery modality comprises an antibody-drug conjugate, a nanoparticle, a chemical conjugate, or a combination thereof.

220. The method of claim 219, wherein the nanoparticle comprises a lipid nanoparticle.

221. The method of claim 219, wherein the chemical conjugate comprises *N*-Acetylgalactosamine (GalNAc).

222. The method of any one of claims 219–221, wherein a protein, antibody, peptide, or nucleic acid molecule is conjugated to or incorporated in the nanoparticle or the chemical conjugate.

223. The method of claim 222, wherein the nucleic acid comprises an RNAi molecule.

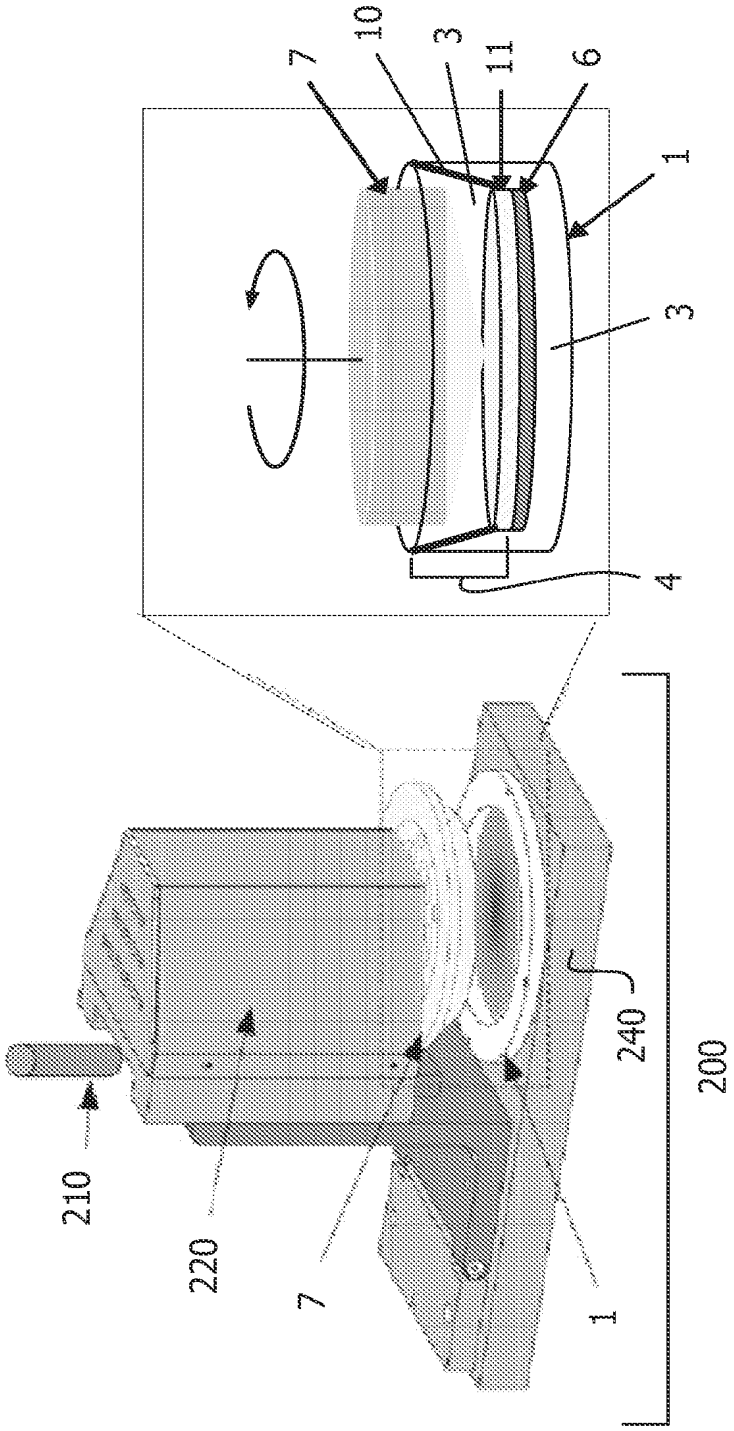
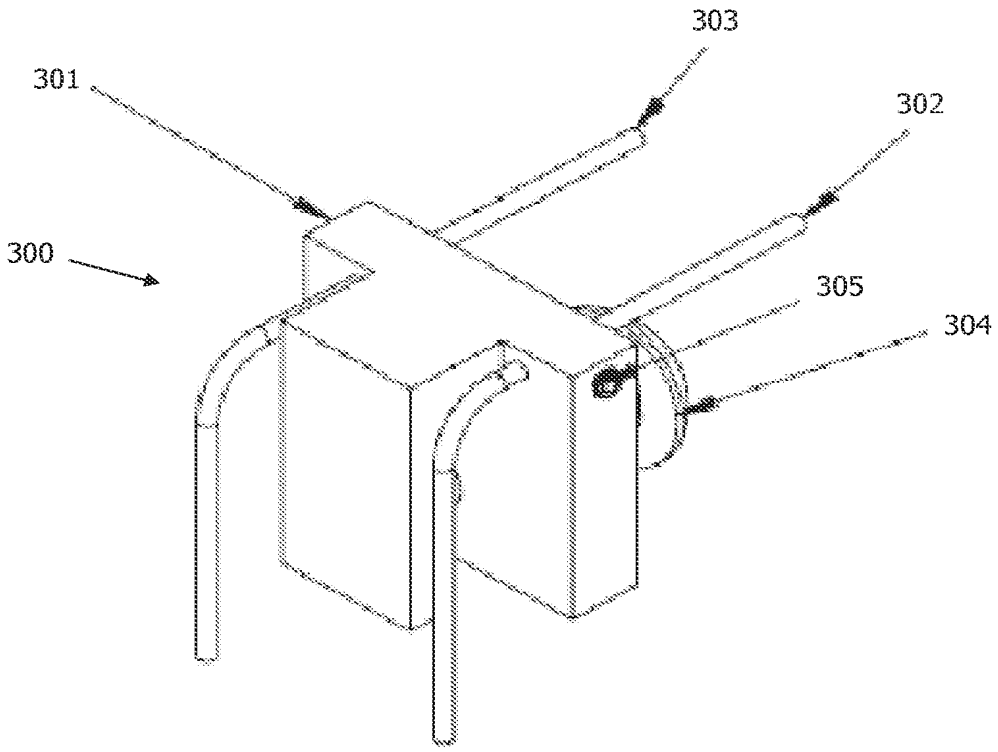


FIG. 1

FIG. 2



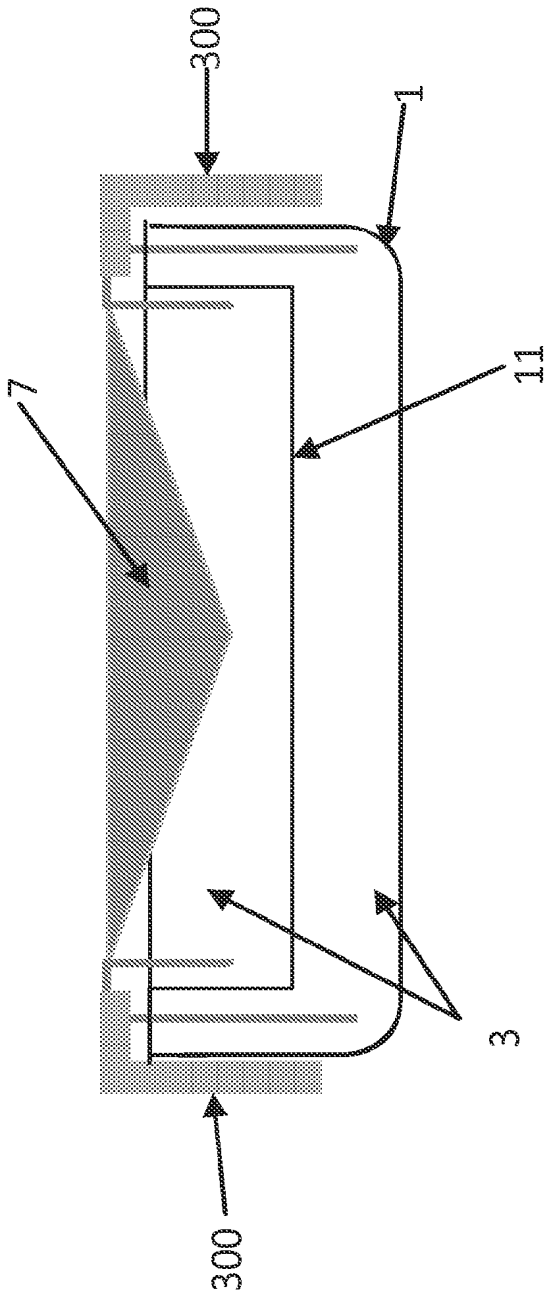


FIG. 3

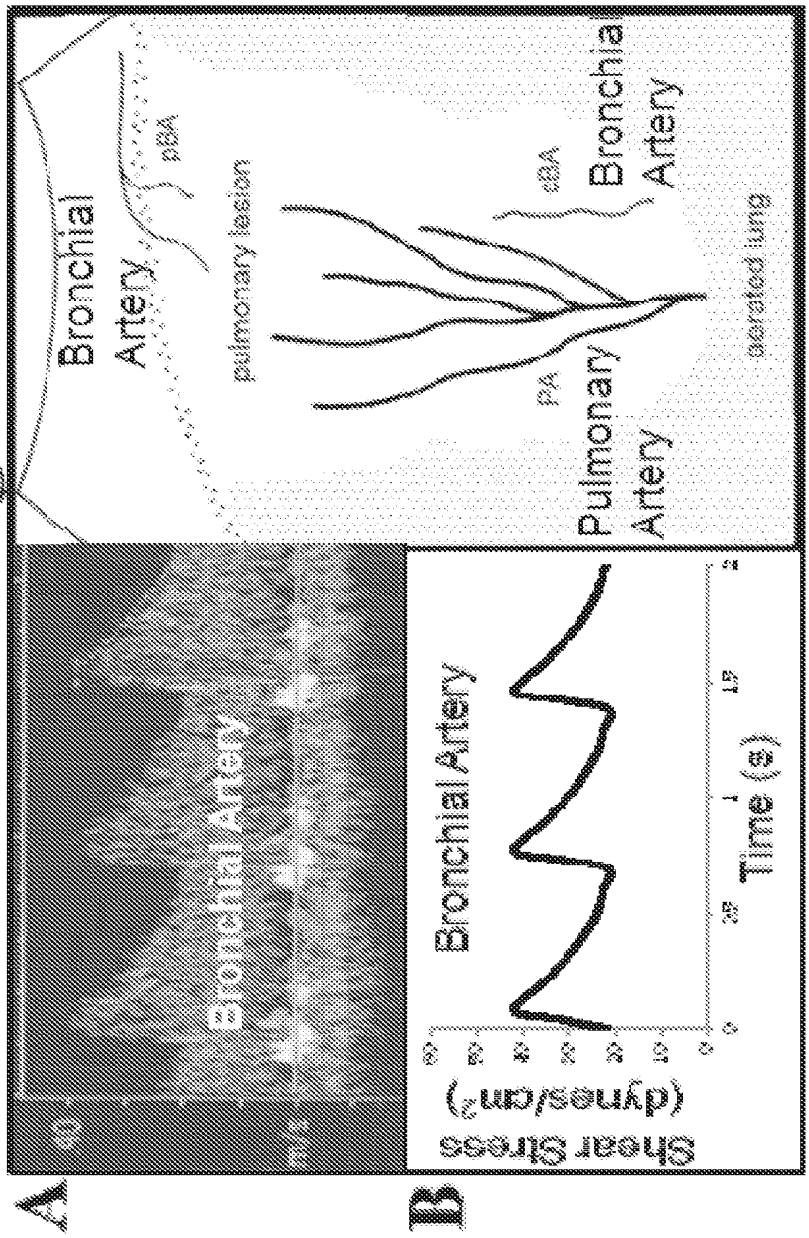
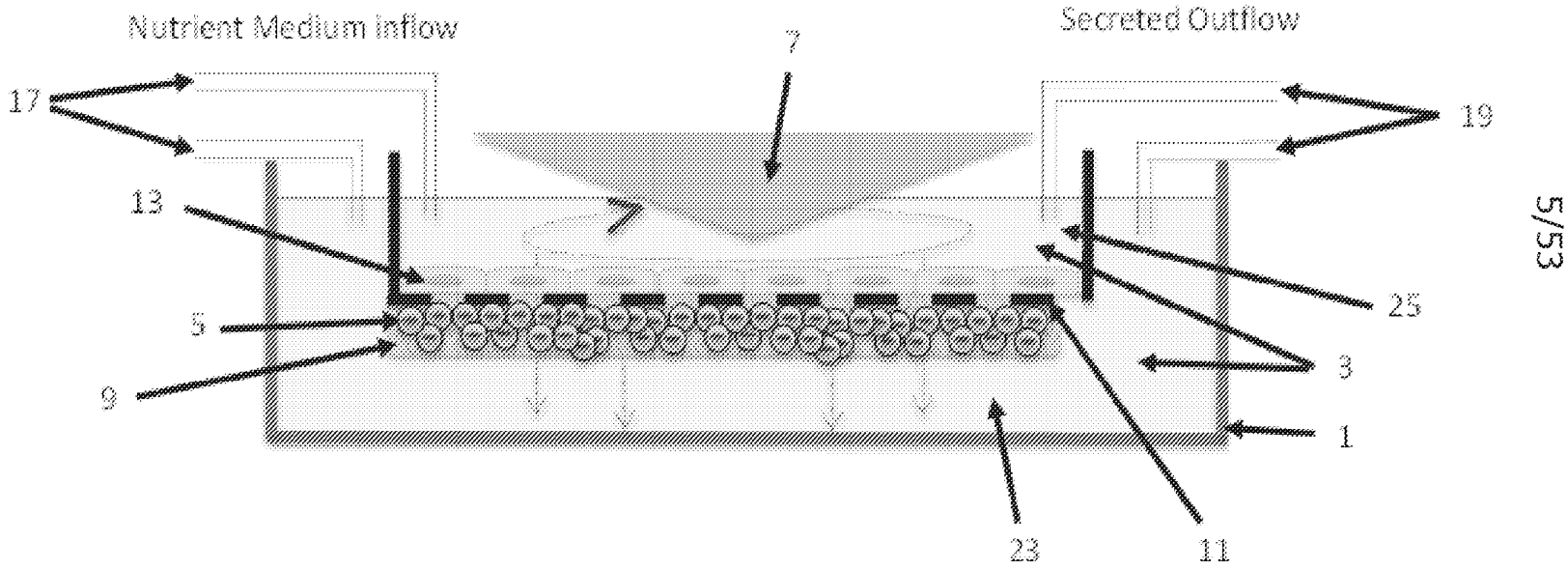

A
Bronchial Artery
B
Shear Stress (dynes/cm²)
Bronchial Artery
Time (s)
C
Bronchial Artery
pBA
pulmonary lesion
PA
Pulmonary Artery
sBA
Bronchial Artery
aerated lung

FIG. 4

FIG. 5


Nutrient Medium Inflow
Secreted Outflow
17
7
19
13
25
5
3
9
1
23
11

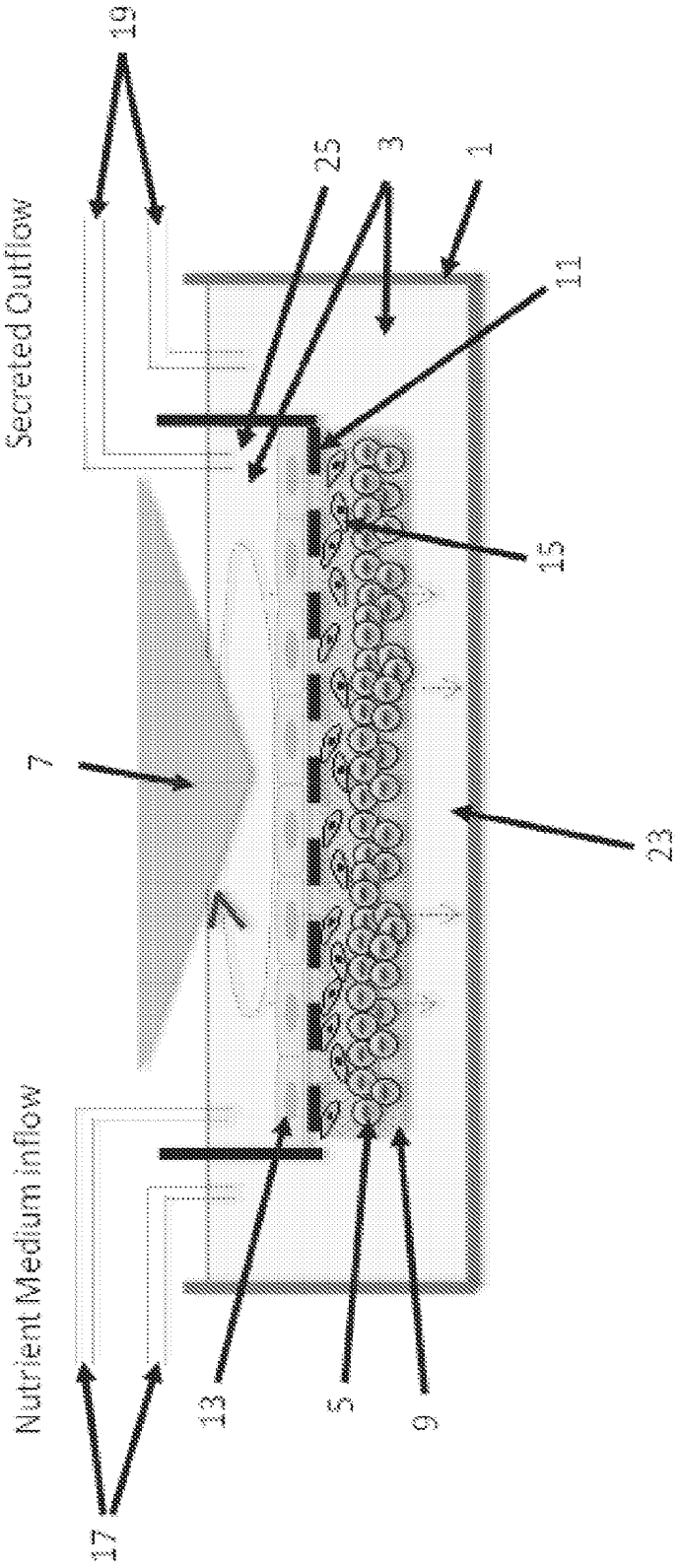

19
25
3
1
11
Secreted Outflow
15
7
23
Nutrient Medium inflow
13
5
9
17

FIG. 6

FIG. 7

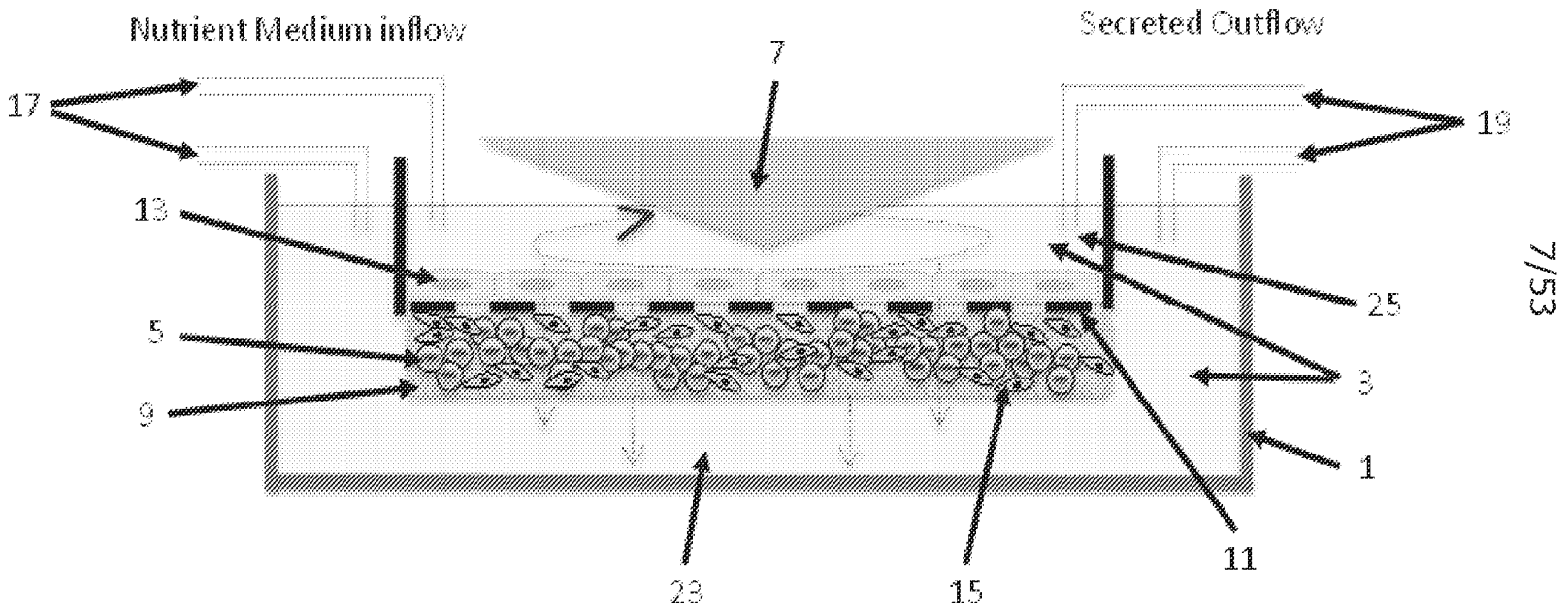

Nutrient Medium inflow
Secreted Outflow
17
7
19
13
7
25
5
3
9
1
23
15
11

FIG. 8

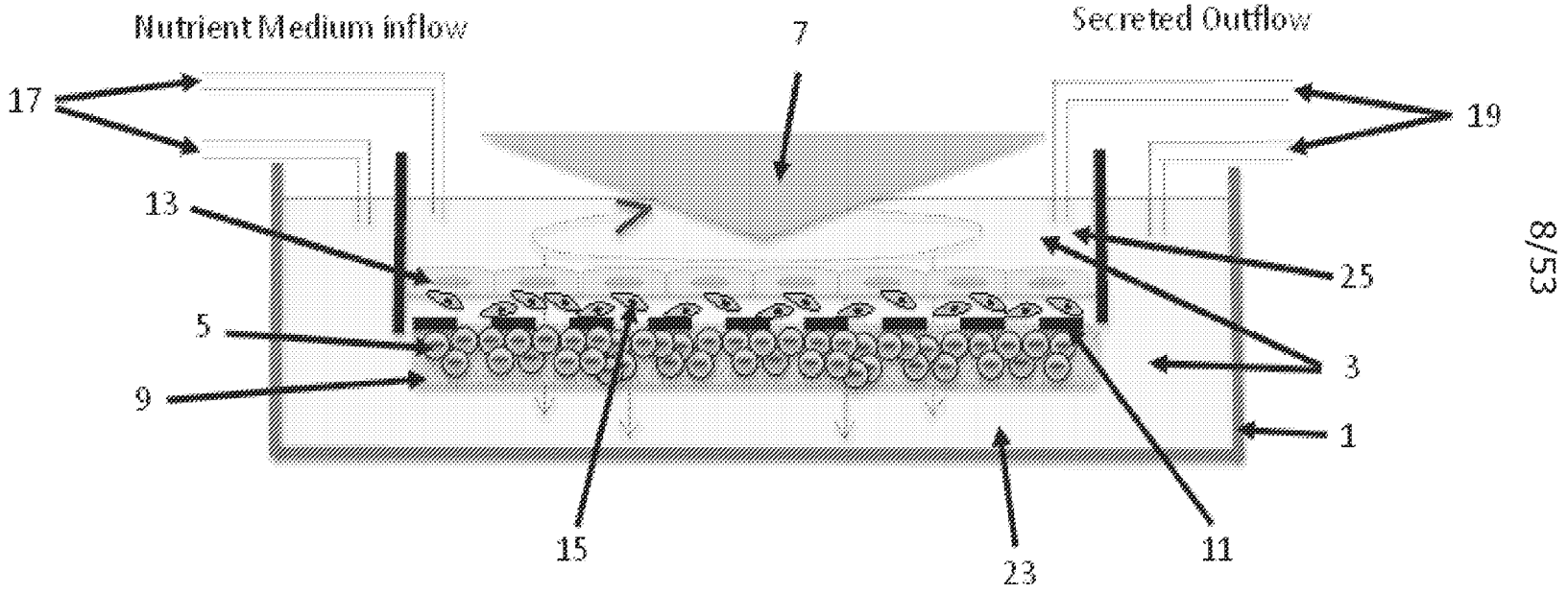

Nutrient Medium inflow
Secreted Outflow
7
17
19
13
25
5
3
9
1
15
23
11

FIG. 9

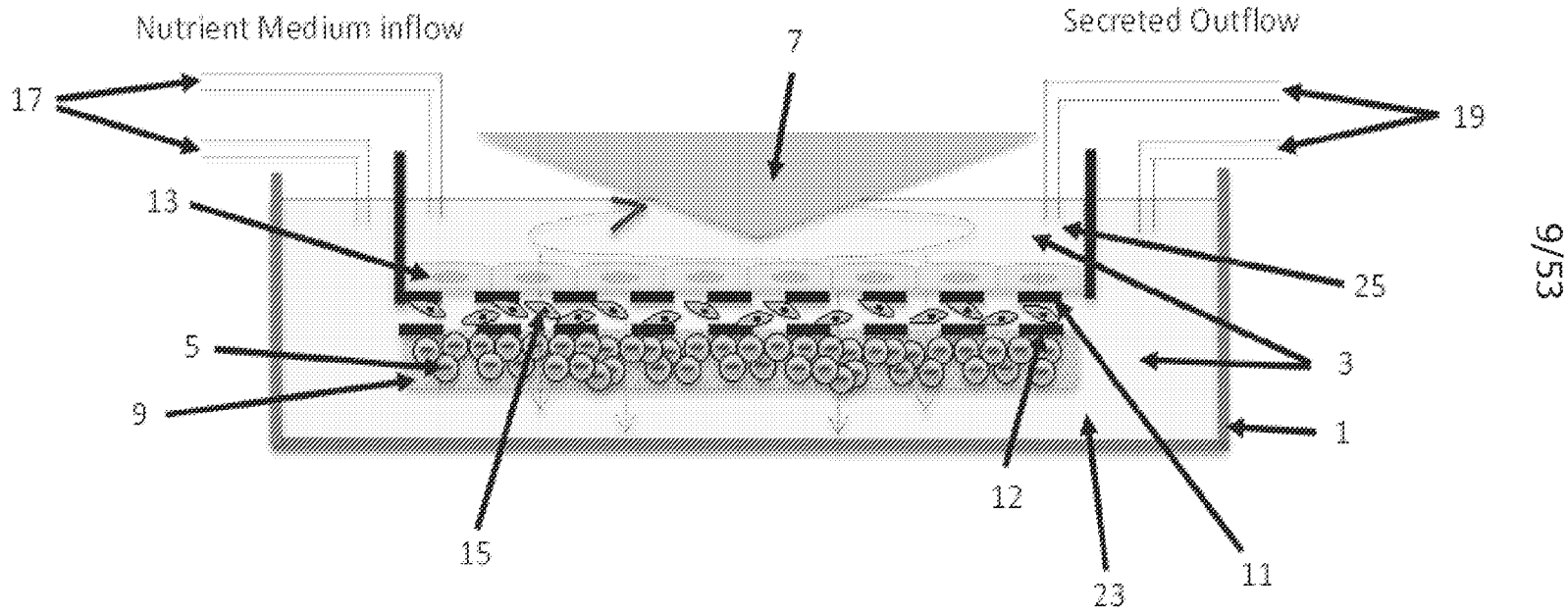
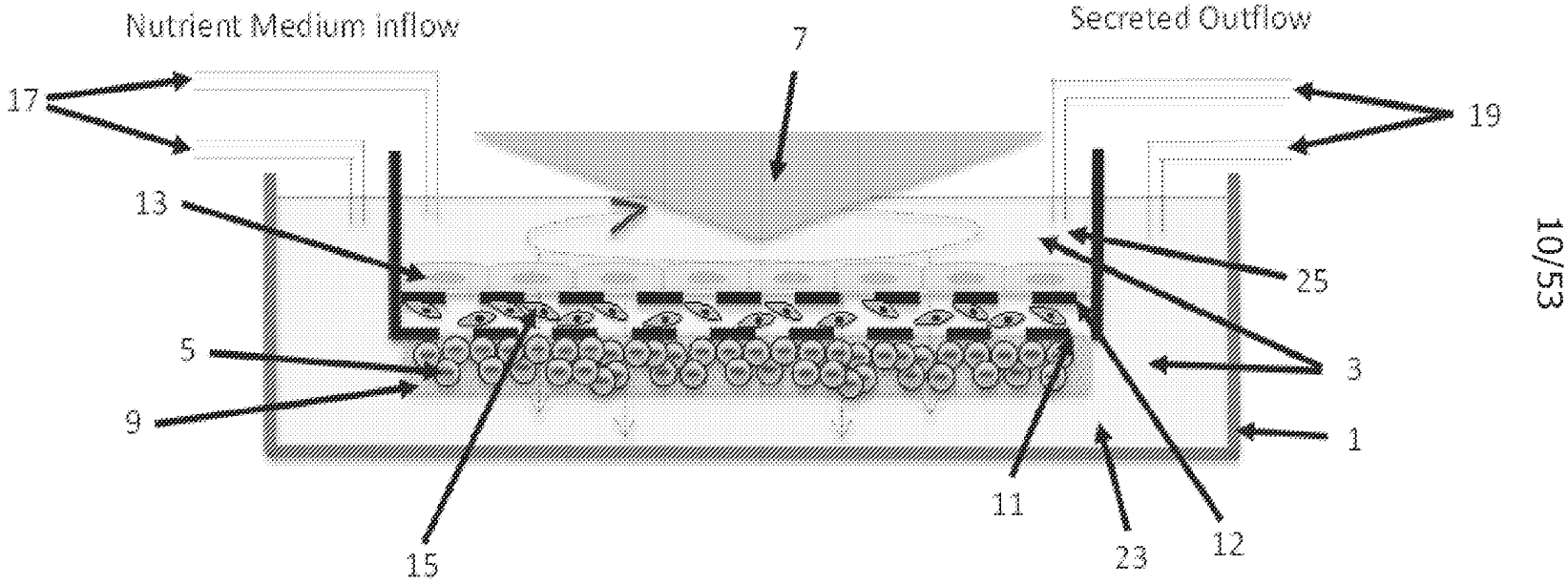

Nutrient Medium inflow
Secreted Outflow
7
17
19
13
25
5
3
9
1
12
15
23
11

FIG. 10


Nutrient Medium inflow
Secreted Outflow
7
17
19
13
25
5
3
9
1
11
23
12
15

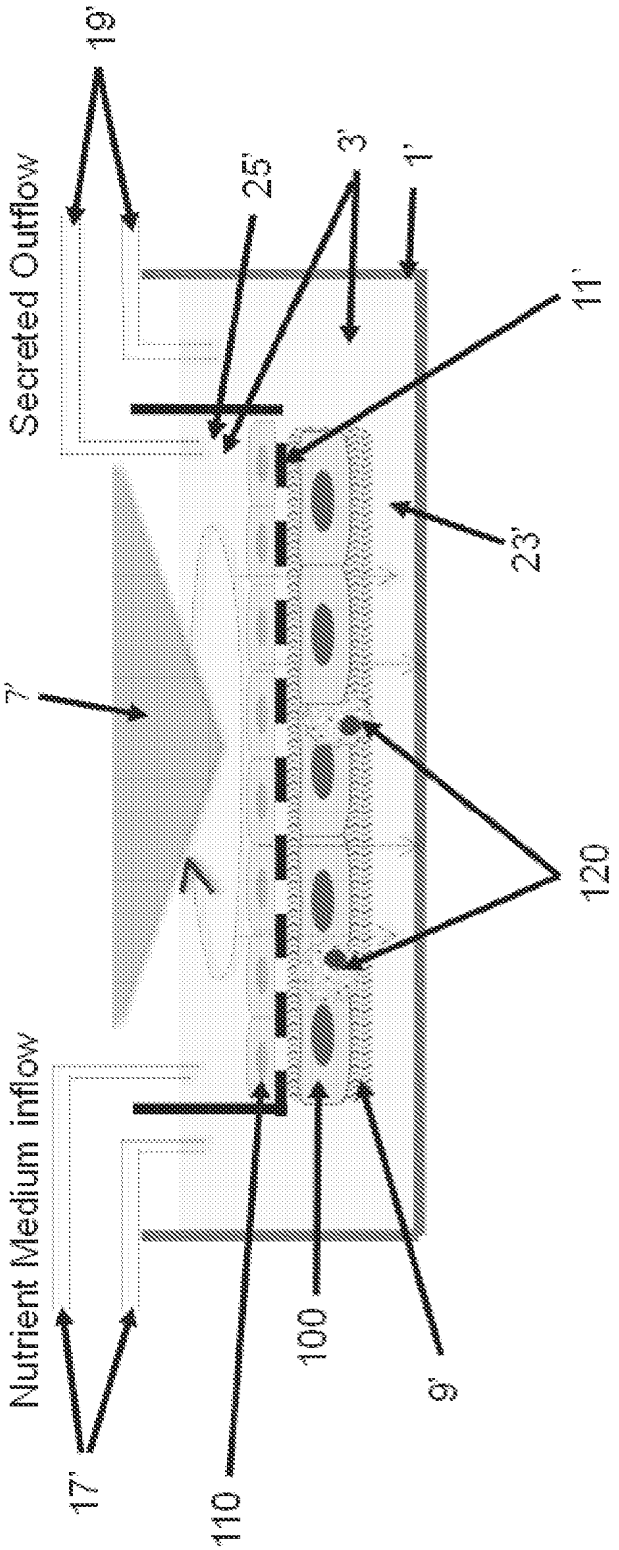

Secreted Outflow
Nutrient Medium inflow
19'
25'
3'
1'
11'
23'
7'
120
100
9'
110
17'

FIG. 11

FIG. 12A

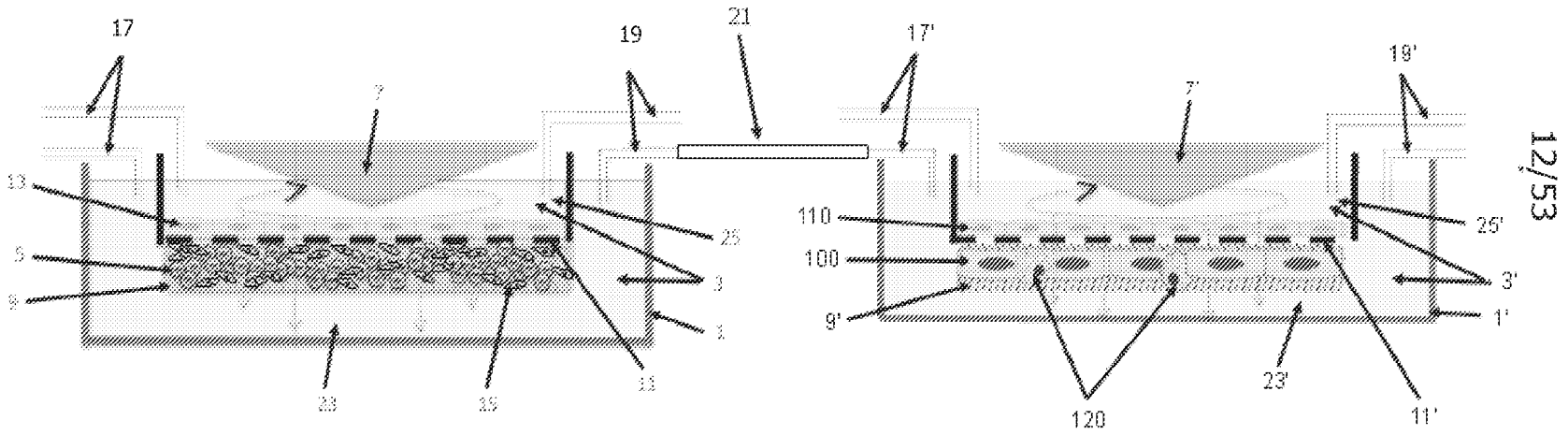


FIG. 12B

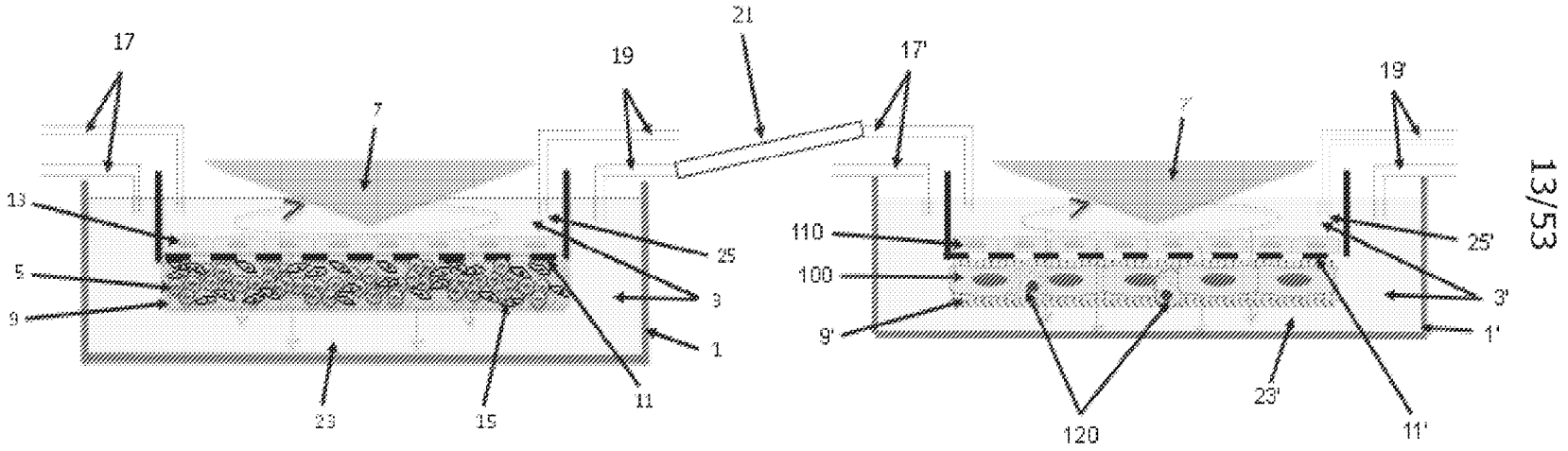


FIG. 12C

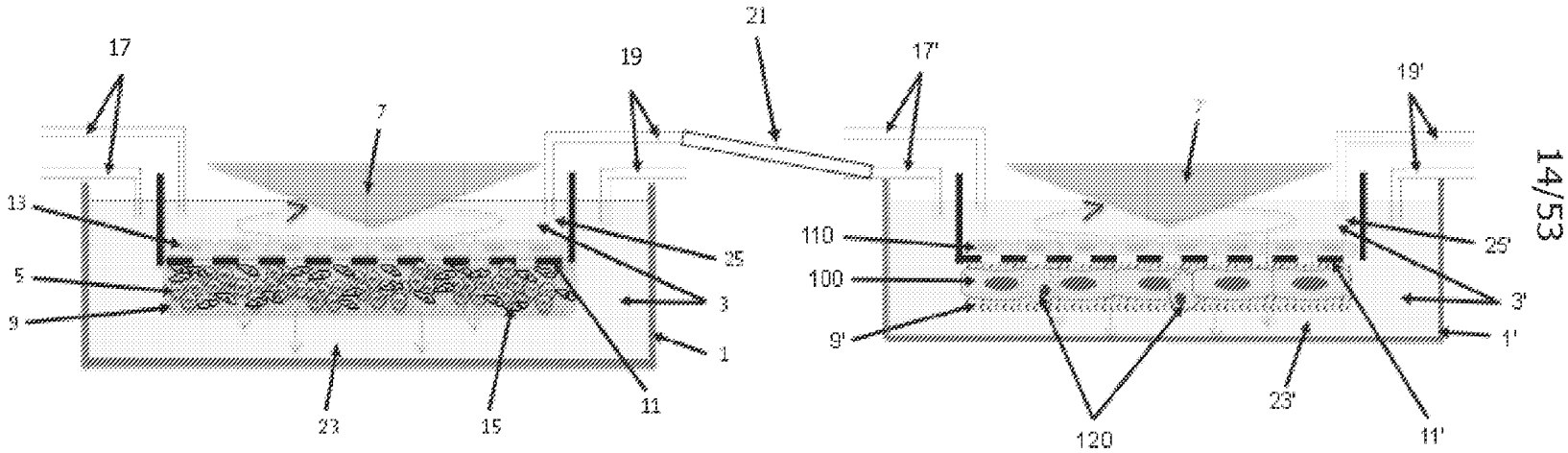


FIG. 12D

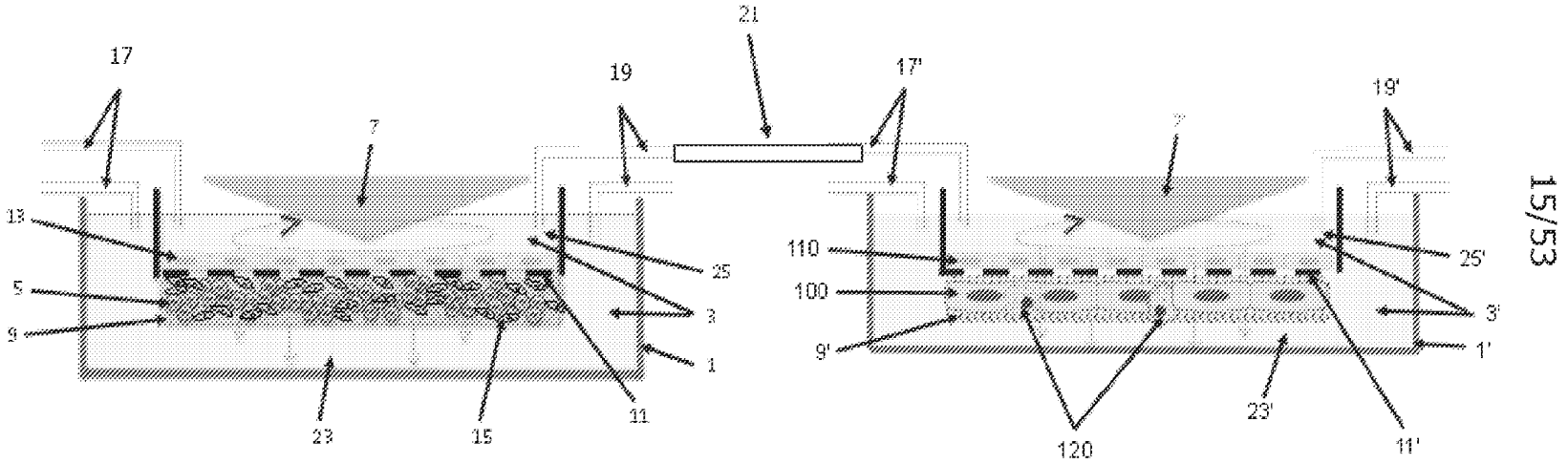
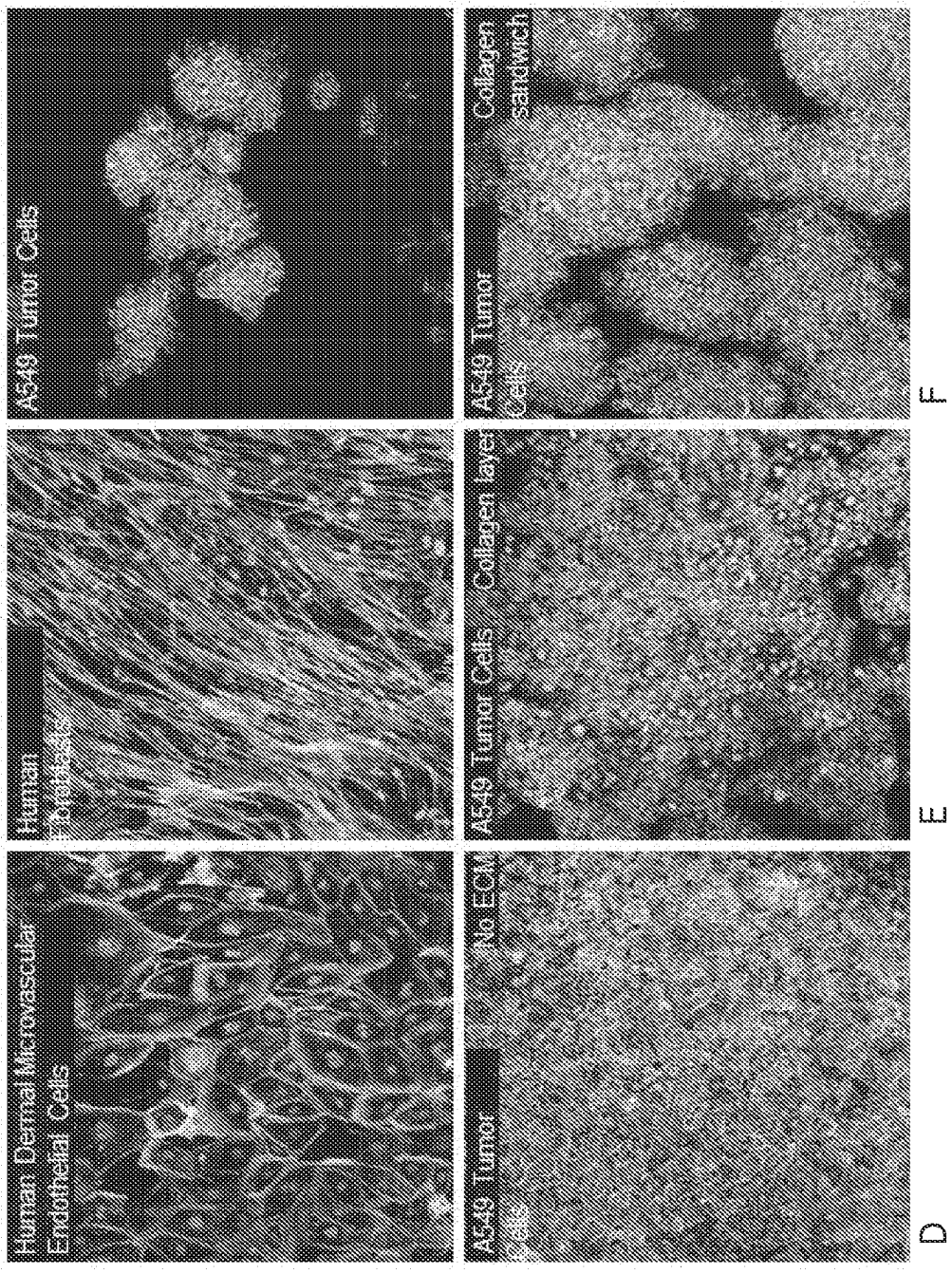


FIG. 13


A
Human Dermal Microvascular Endothelial Cells
B
Human Angioblasts
C
A549 Tumor Cells
D
A549 Tumor Cells
No ECM
E
A549 Tumor Cells
Collagen layer
F
A549 Tumor Cells
Collagen sandwich

Cell Number
50000
100000
150000
0
Days
0
1
2
3
4
5
6
7
Phase I
Phase II
Static
In vitro tumor microenvironment

FIG. 14A

FIG. 14B

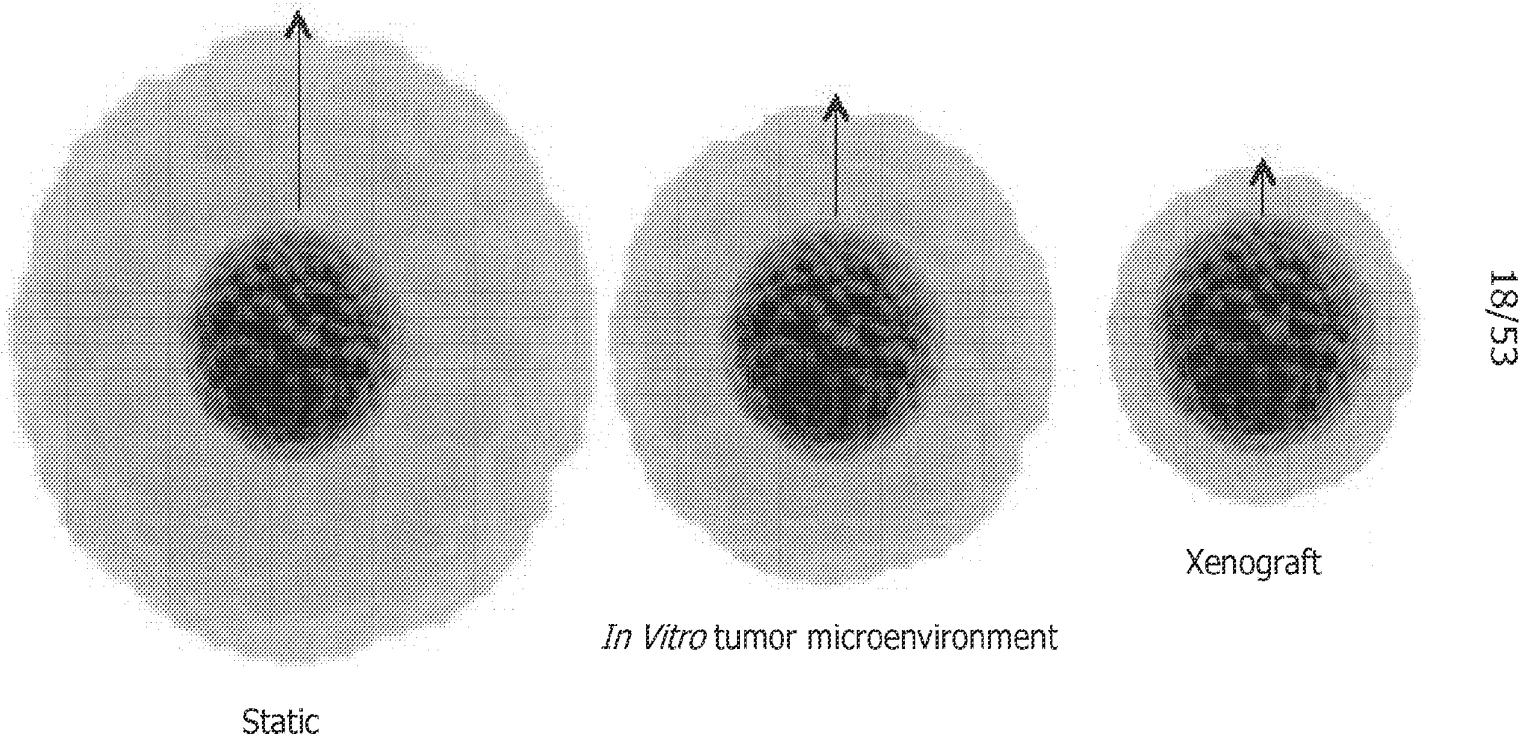


FIG. 14C

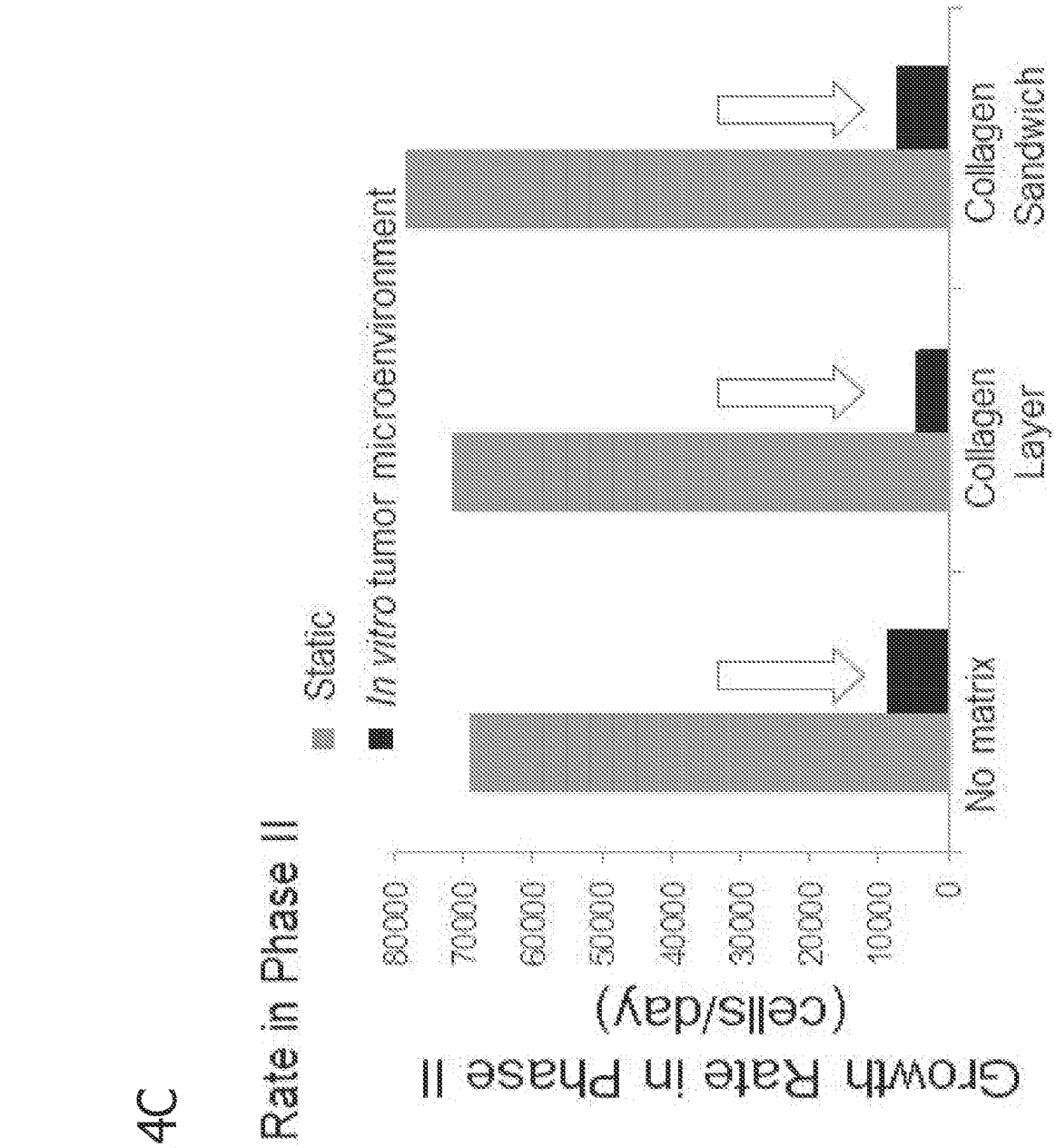
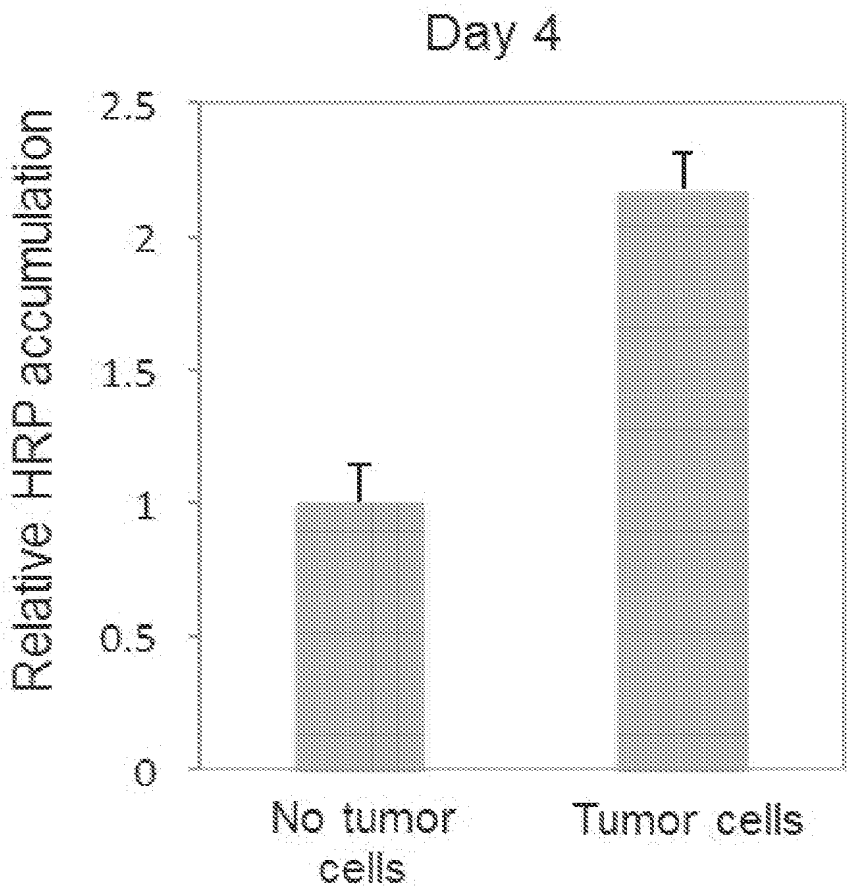

Rate in Phase II
Static
In vitro tumor microenvironment
Growth Rate in Phase II
(cells/day)
80000
70000
60000
50000
40000
30000
20000
10000
0
No matrix
Collagen Layer
Collagen Sandwich

FIG. 15



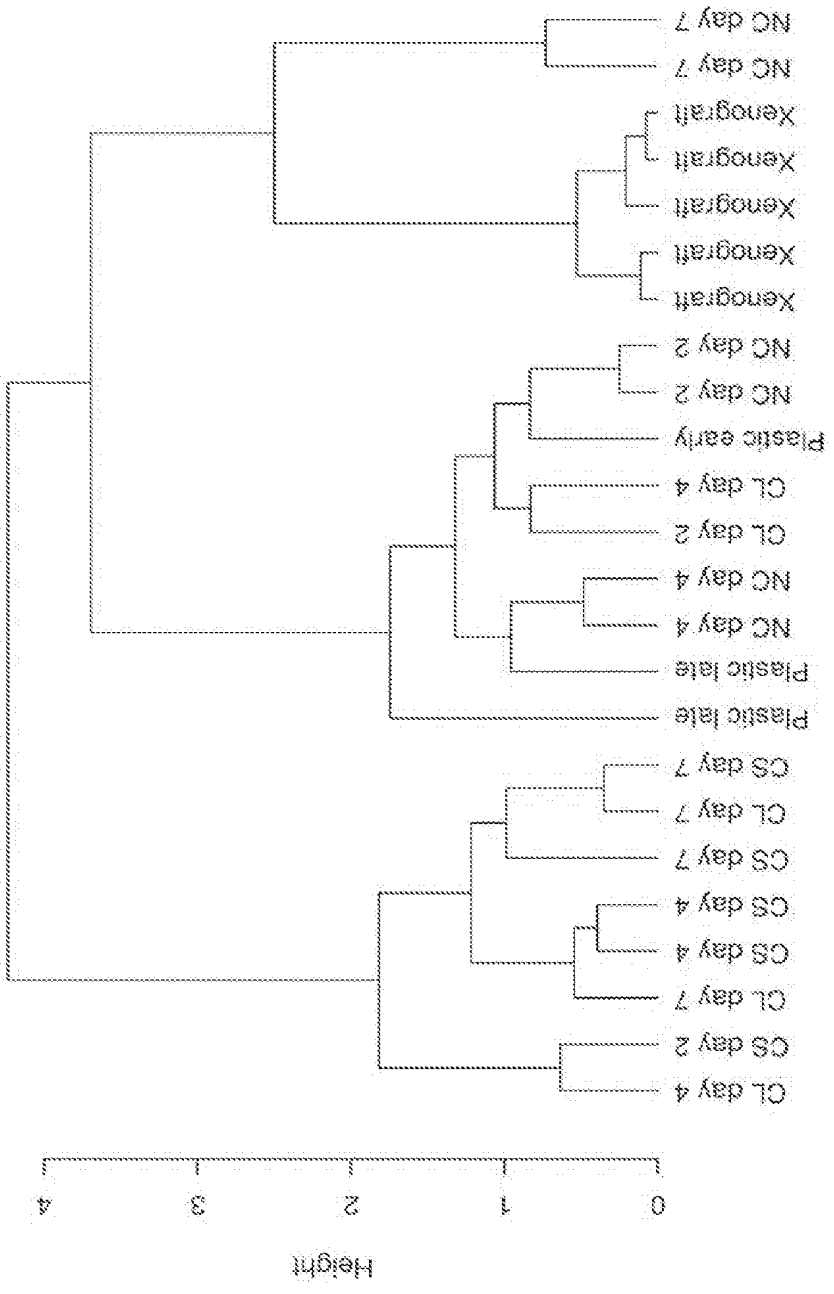

CL day 4
CS day 2
CL day 7
CS day 4
CS day 4
CS day 7
CL day 7
CS day 7
Plastic late
Plastic late
NC day 4
NC day 4
CL day 2
CL day 4
Plastic early
NC day 2
NC day 2
Xenograft
Xenograft
Xenograft
Xenograft
Xenograft
NC day 7
NC day 7
0
1
2
3
4
Height

FIG. 16A

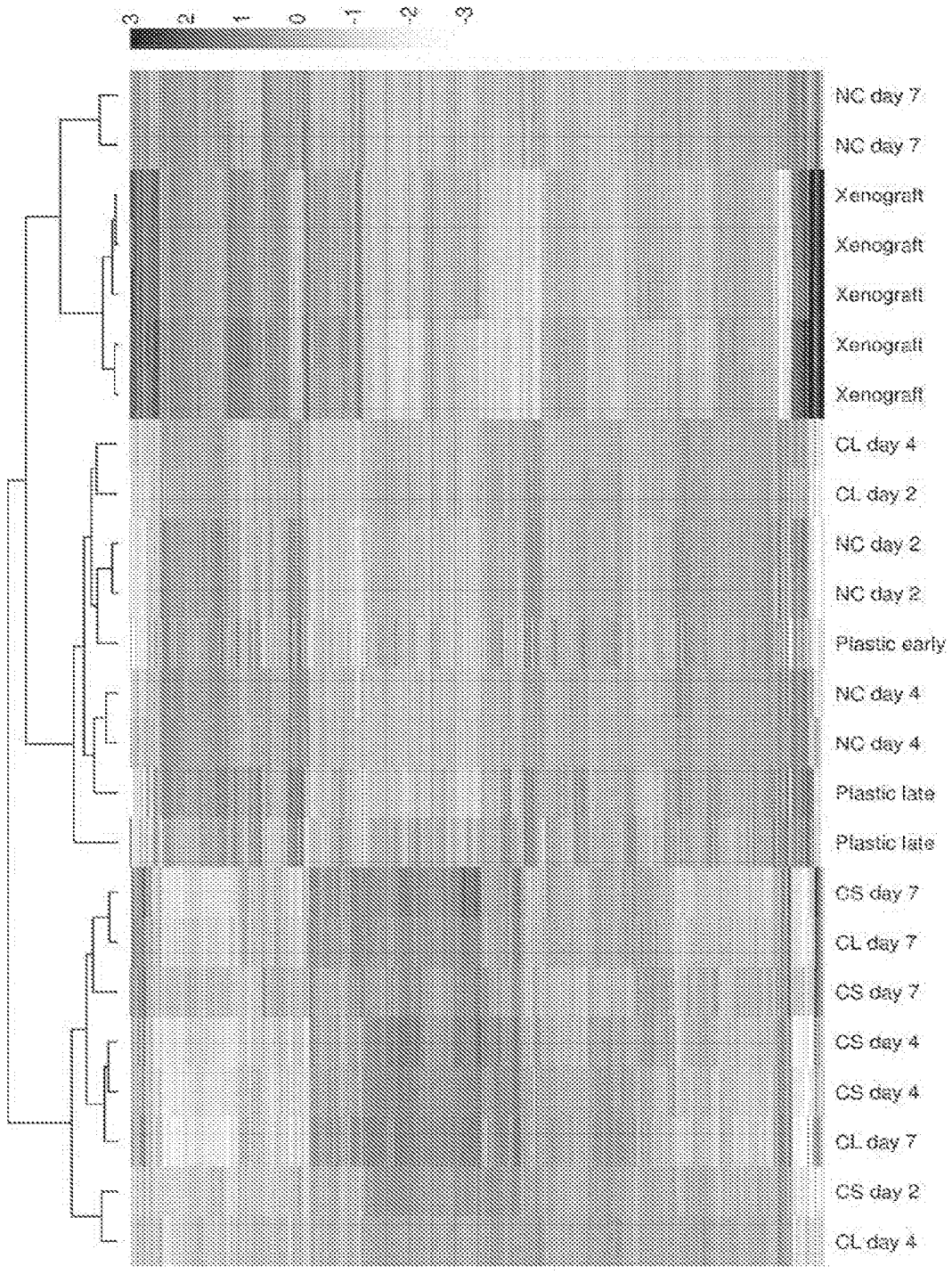

3
2
1
0
-1
-2
-3
NC day 7
NC day 7
Xenograft
Xenograft
Xenograft
Xenograft
Xenograft
CL day 4
CL day 2
NC day 2
NC day 2
Plastic early
NC day 4
NC day 4
Plastic late
Plastic late
CS day 7
CL day 7
CS day 7
CS day 4
CS day 4
CL day 7
CS day 2
CL day 4

FIG. 16B

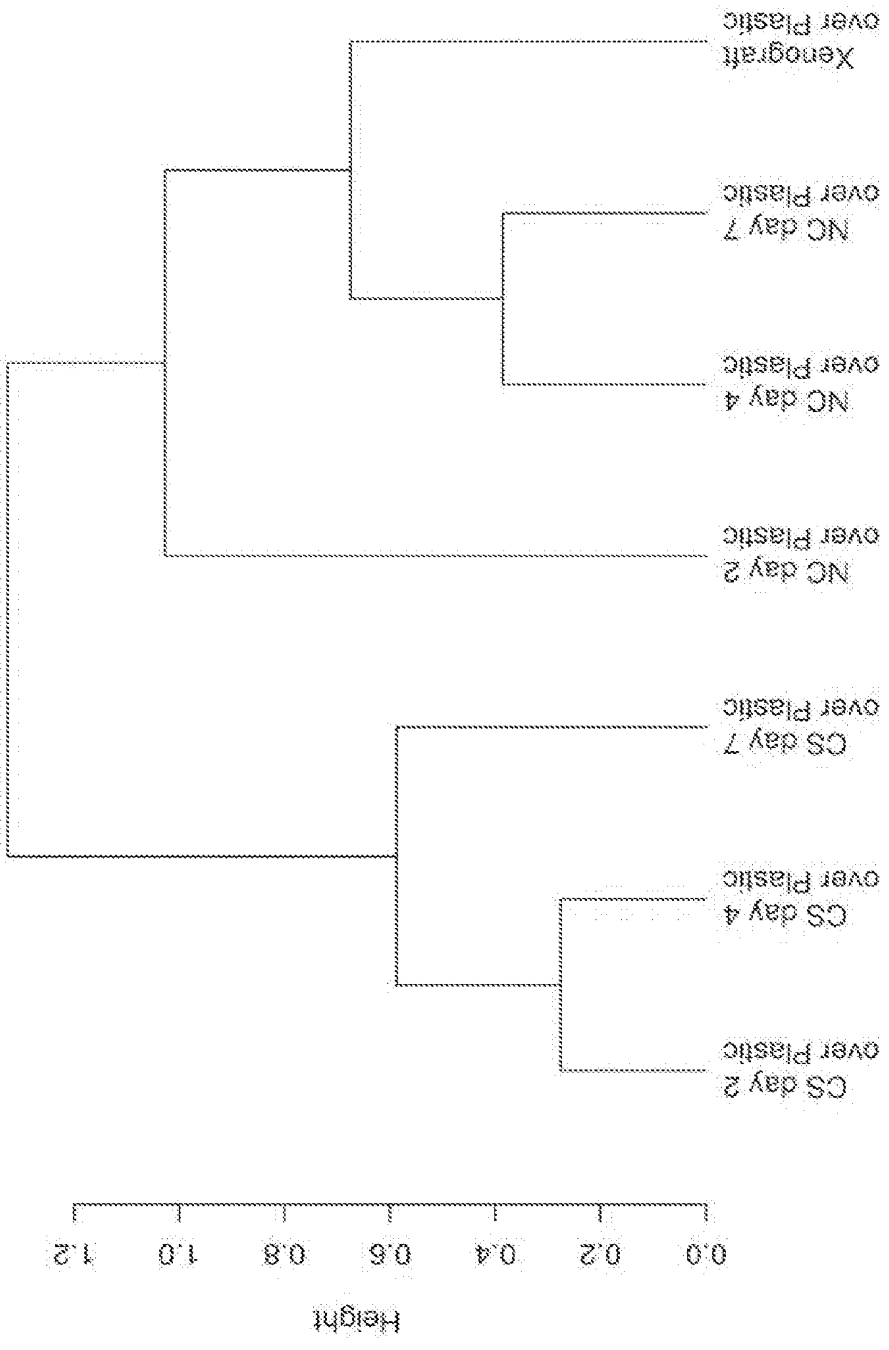
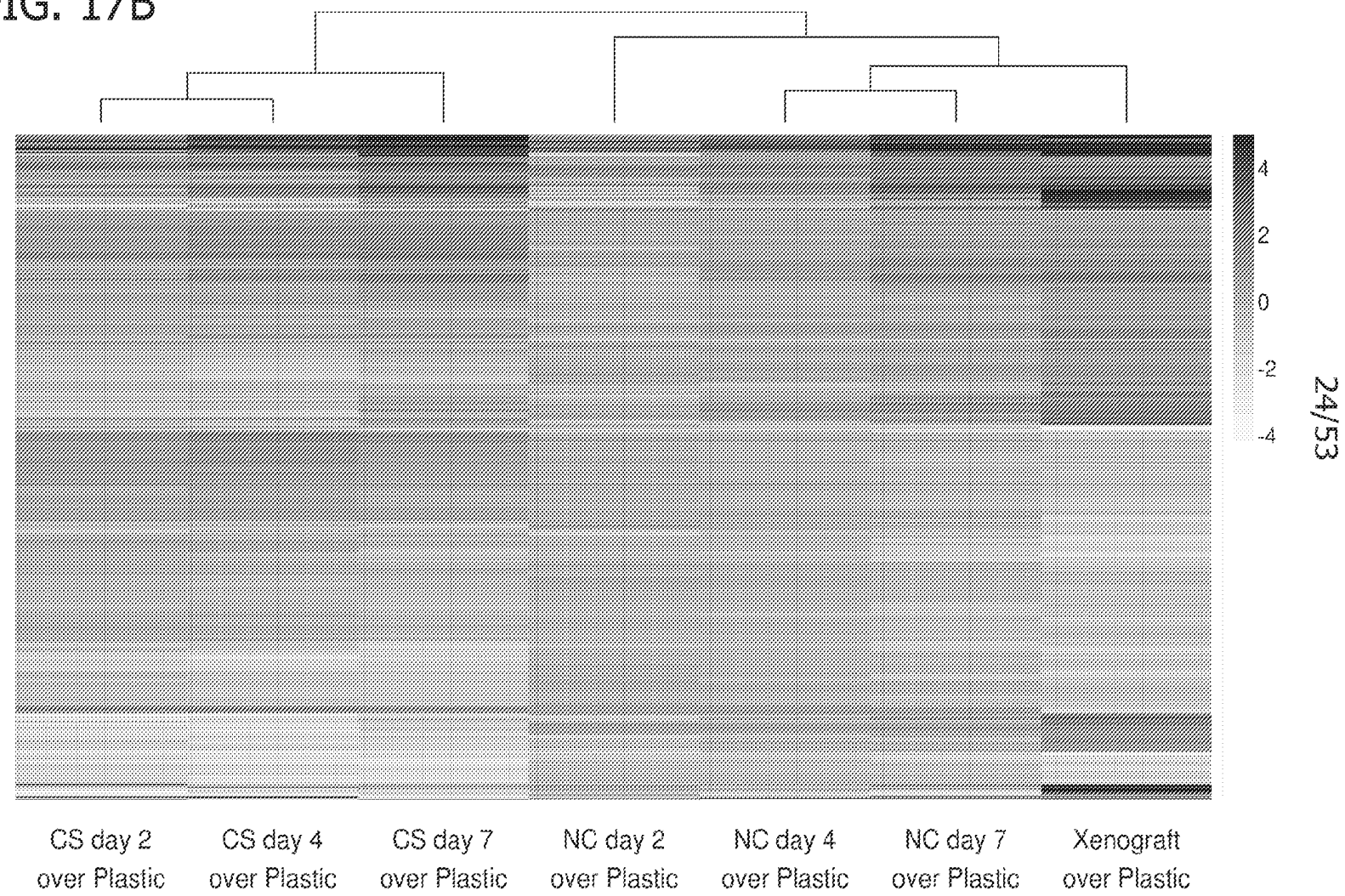

Xenograft over Plastic
NC day 7 over Plastic
NC day 4 over Plastic
NC day 2 over Plastic
CS day 7 over Plastic
CS day 4 over Plastic
CS day 2 over Plastic
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Height

FIG. 17A

FIG. 17B


4
2
0
-2
-4
CS day 2 over Plastic
CS day 4 over Plastic
CS day 7 over Plastic
NC day 2 over Plastic
NC day 4 over Plastic
NC day 7 over Plastic
Xenograft over Plastic

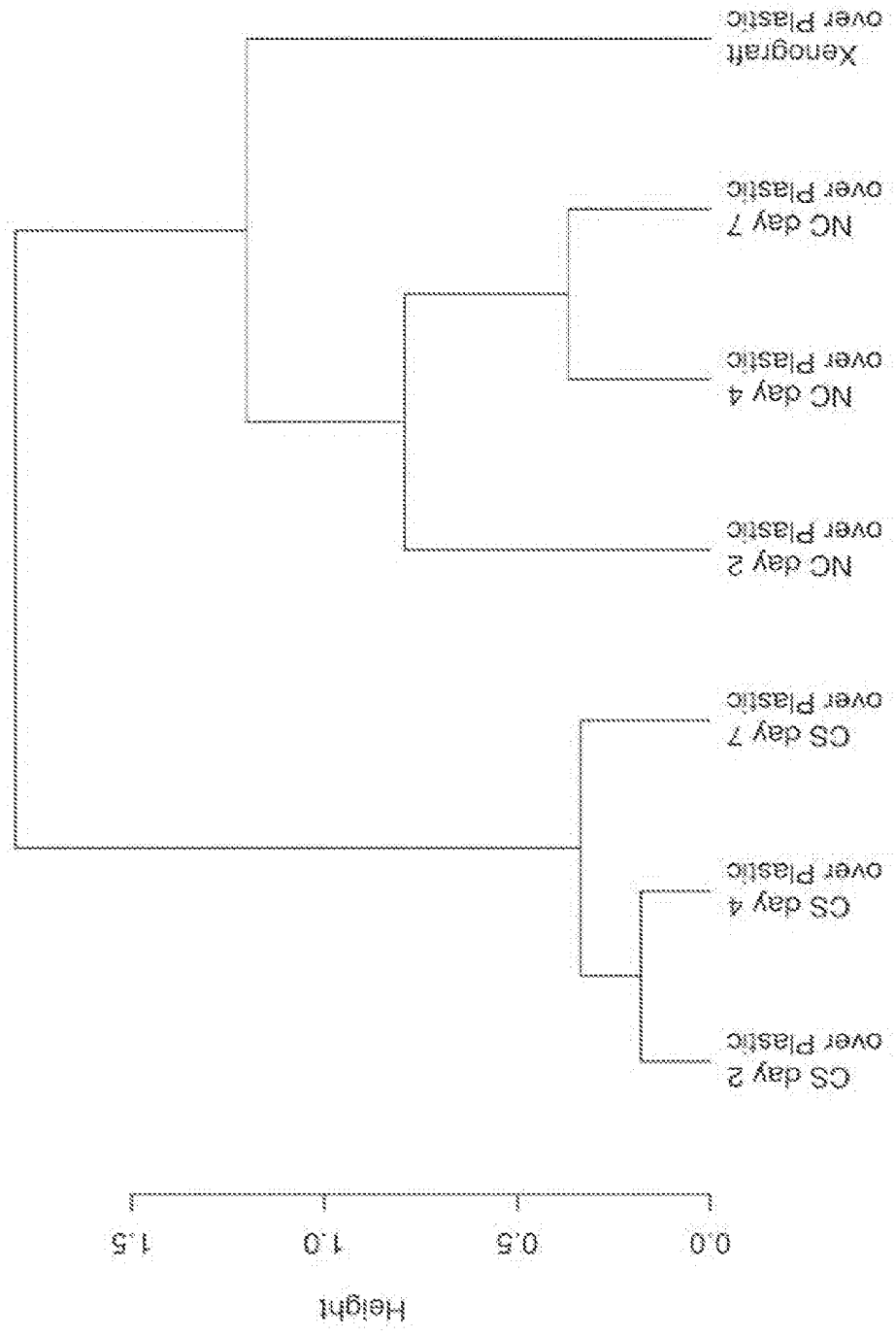

Xenograft over Plastic
NC day 7 over Plastic
NC day 4 over Plastic
NC day 2 over Plastic
CS day 7 over Plastic
CS day 4 over Plastic
CS day 2 over Plastic
0.0
0.5
1.0
1.5
Height

FIG. 18A

FIG. 18B

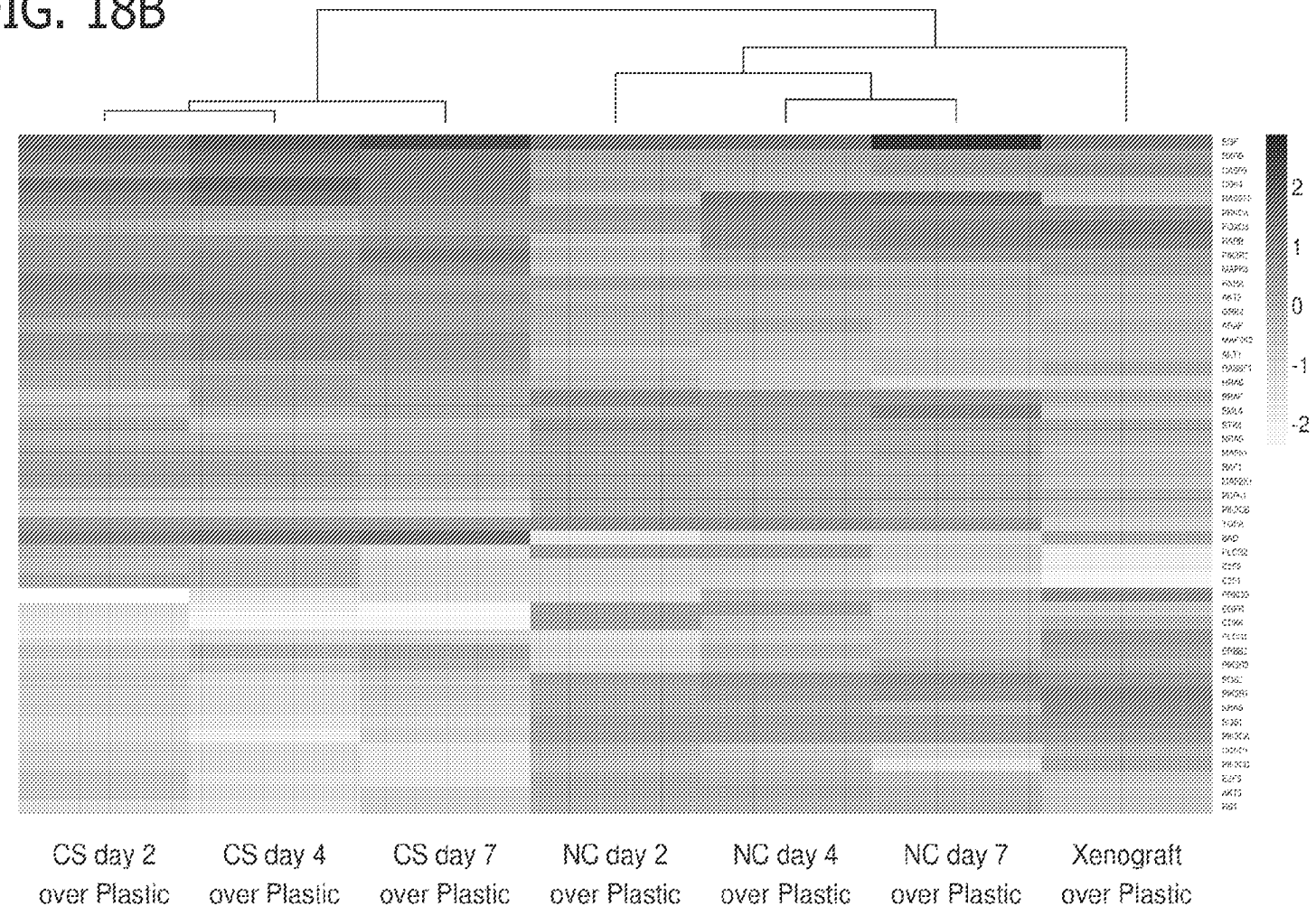

2
1
0
-1
-2
CS day 2 over Plastic
CS day 4 over Plastic
CS day 7 over Plastic
NC day 2 over Plastic
NC day 4 over Plastic
NC day 7 over Plastic
Xenograft over Plastic

FIG. 19A

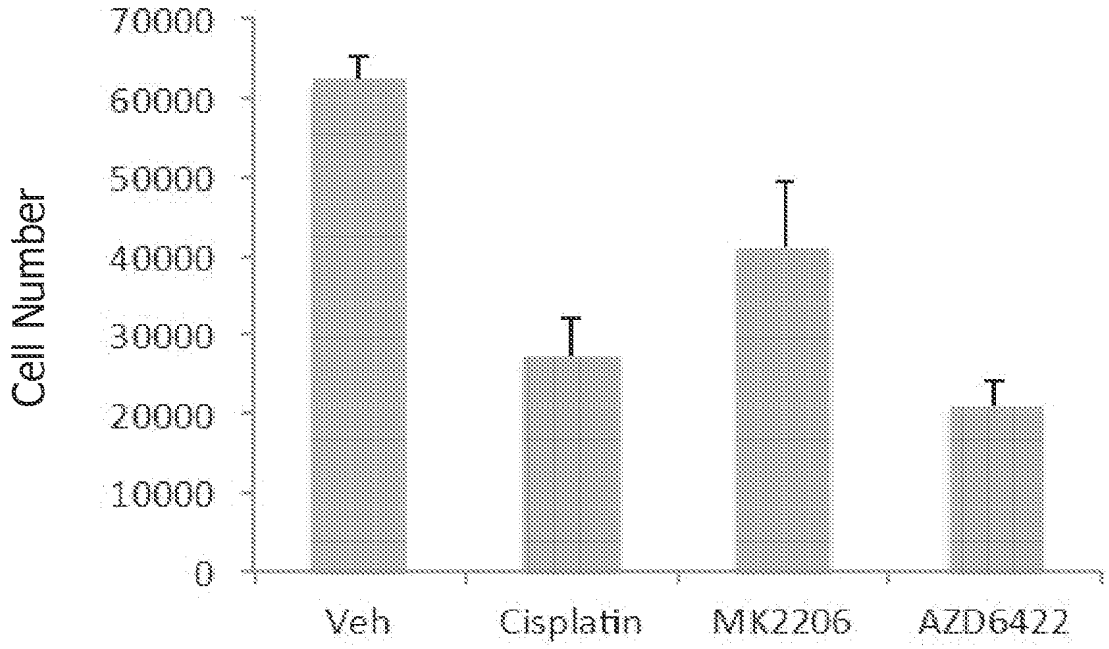


FIG. 19B

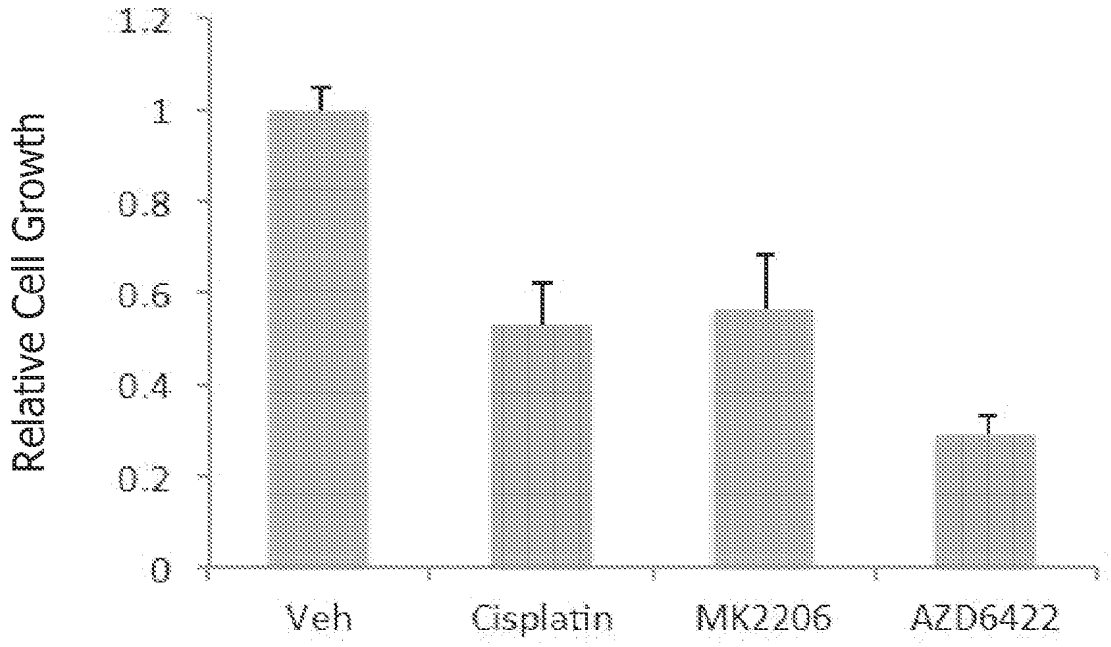


FIG. 20A

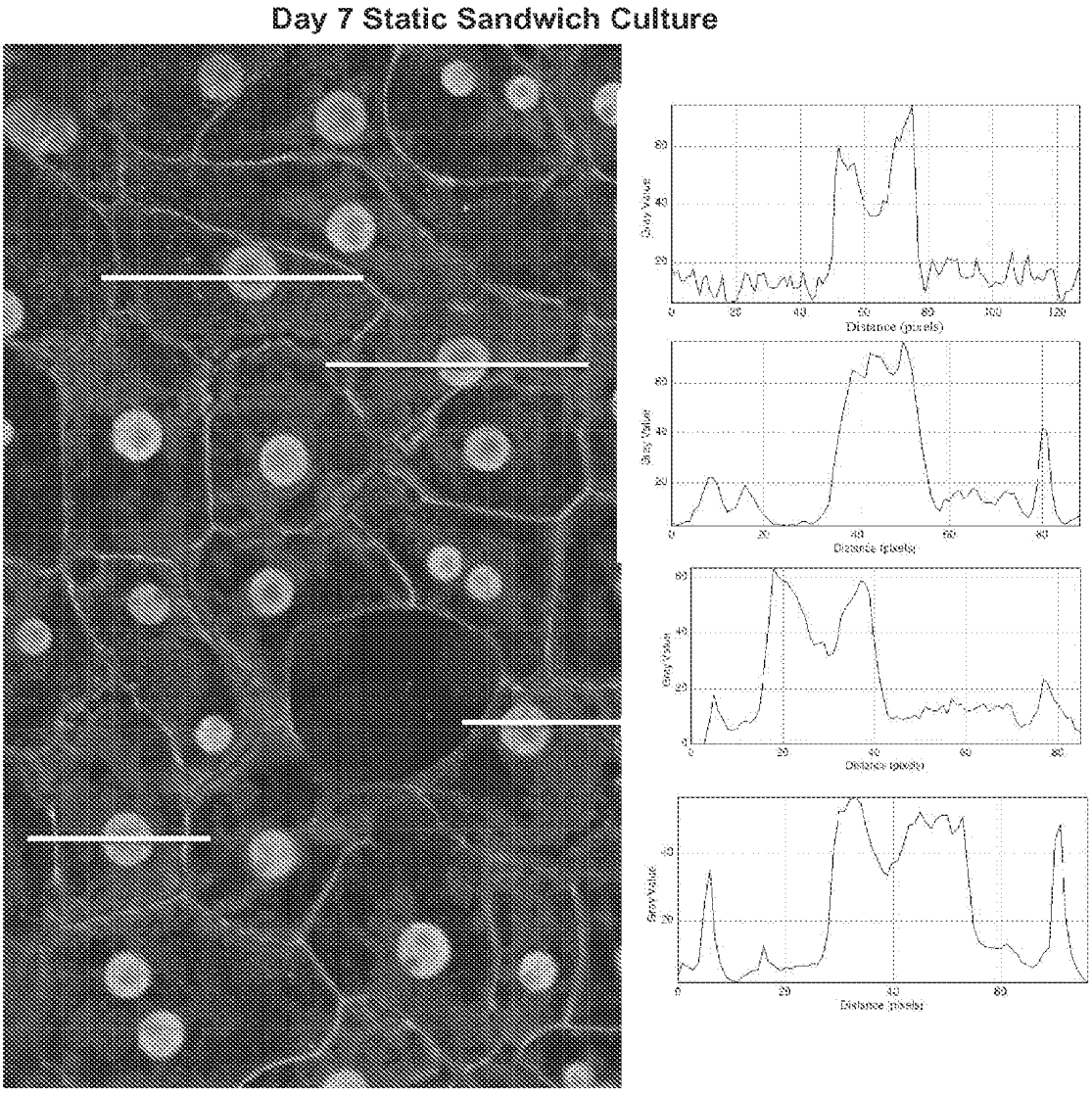
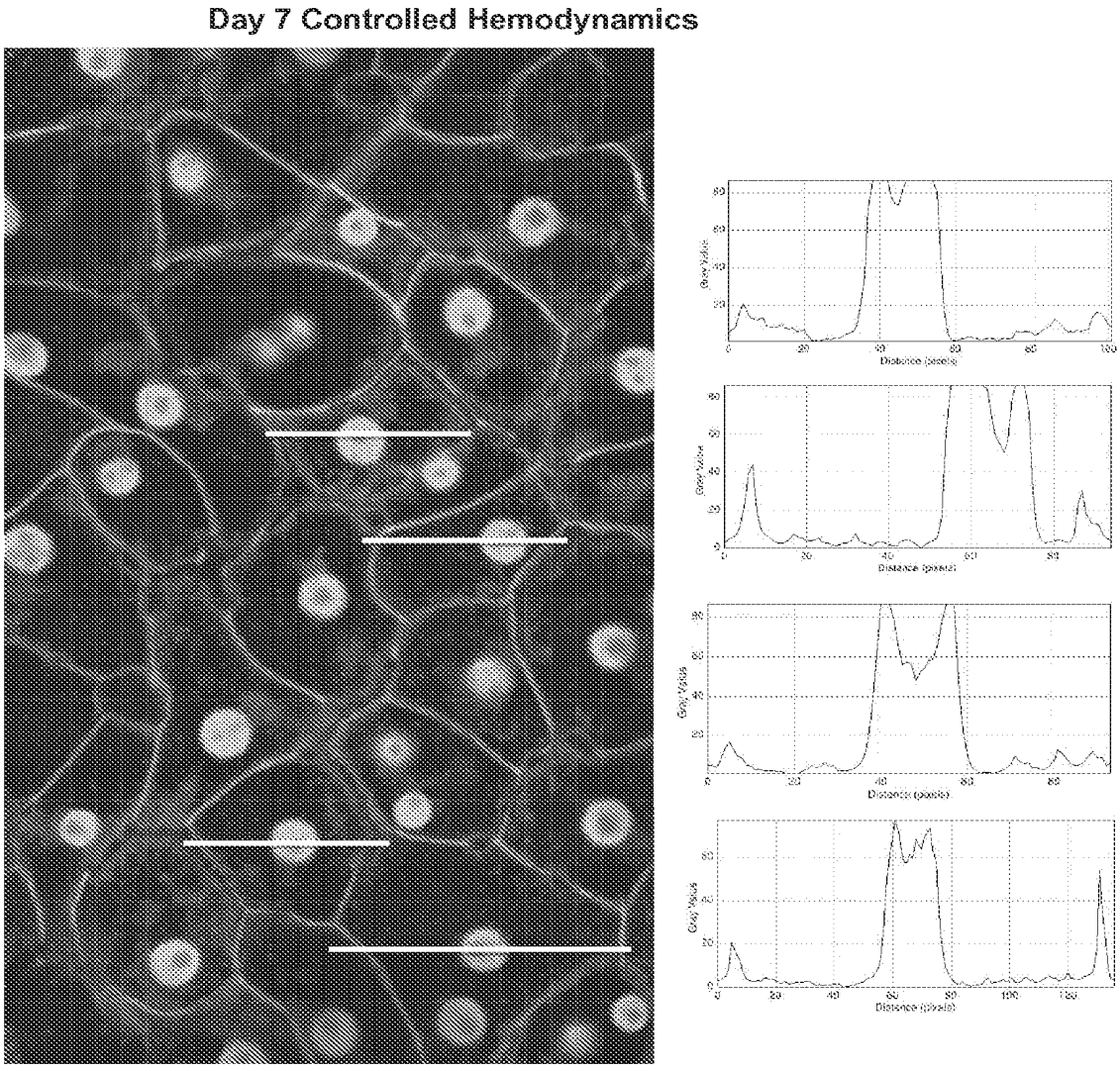

Day 7 Static Sandwich Culture
Gray Value
Distance (pixels)
E-cadherin

FIG. 20B



E-cadherin

FIG. 20C

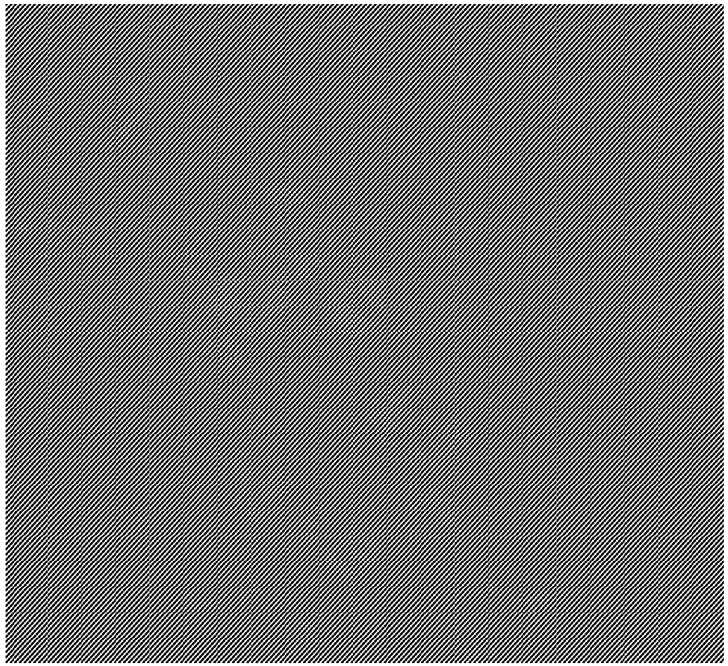


FIG. 20D

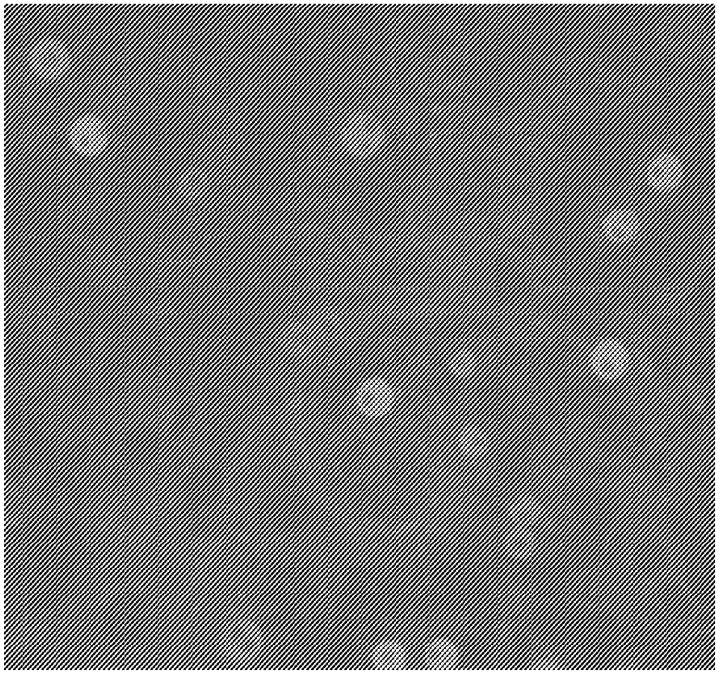
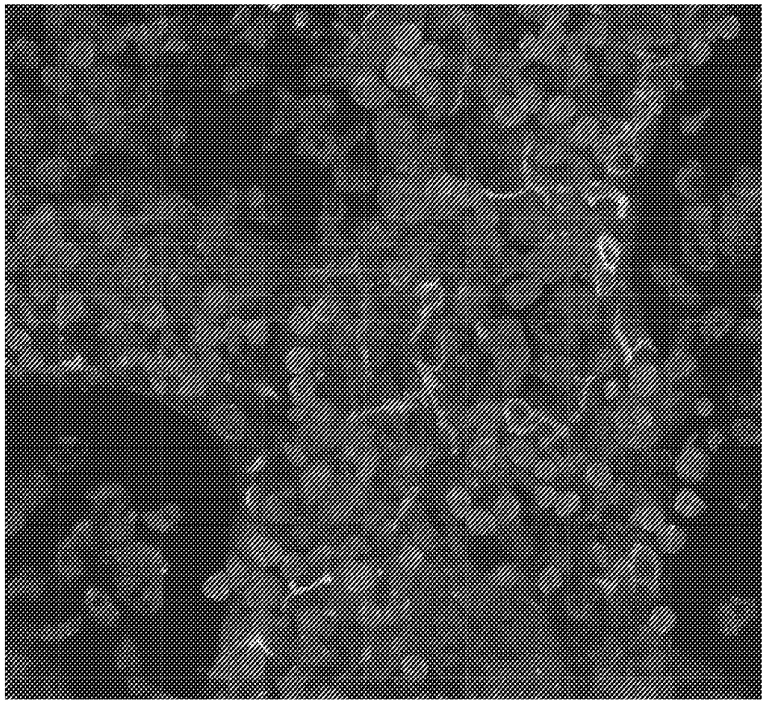


FIG. 20E

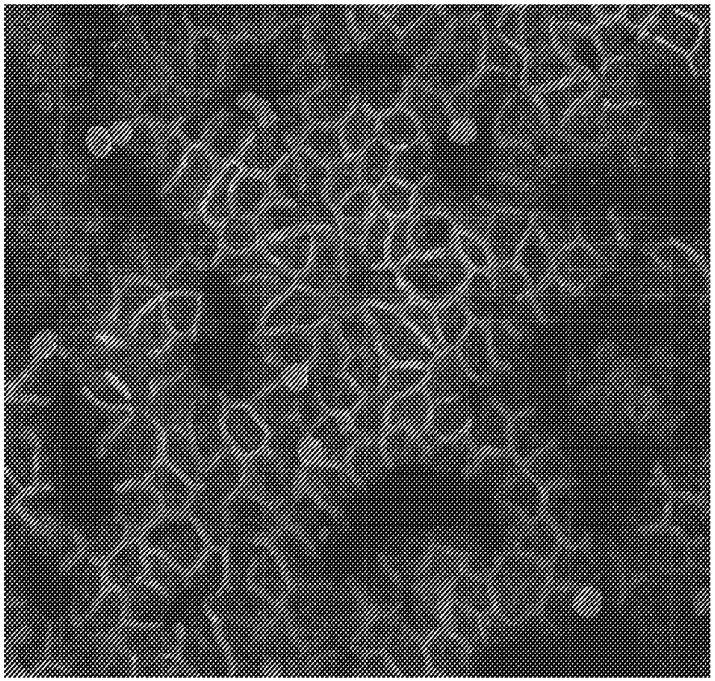
Day 14 Static Sandwich Culture



MRP - 2

FIG. 20F

Day 14 Controlled Hemodynamics



MRP - 2

FIG. 21A

Day 14 Controlled Hemodynamics:

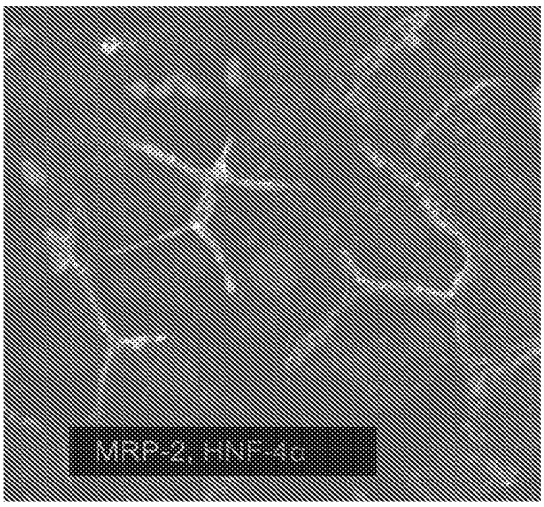


FIG. 21B

In vivo liver:

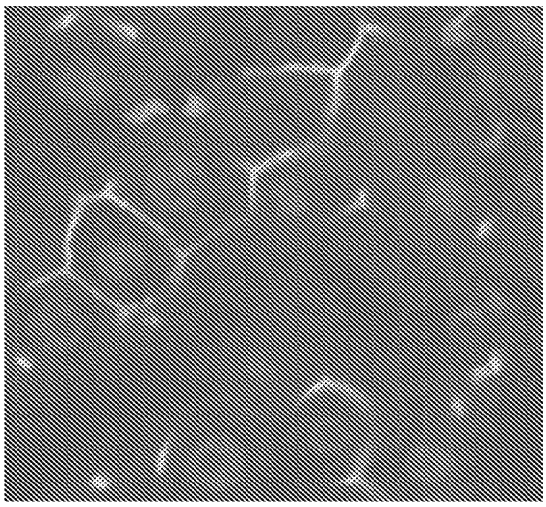


FIG. 21C

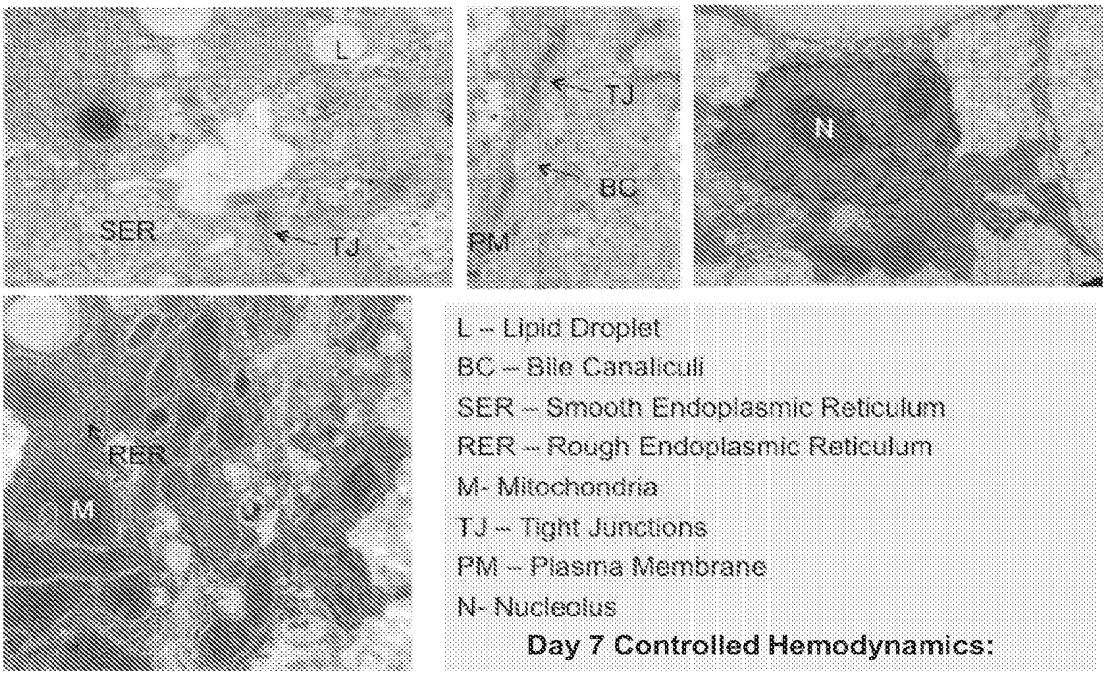


FIG. 22A

FIG. 22B

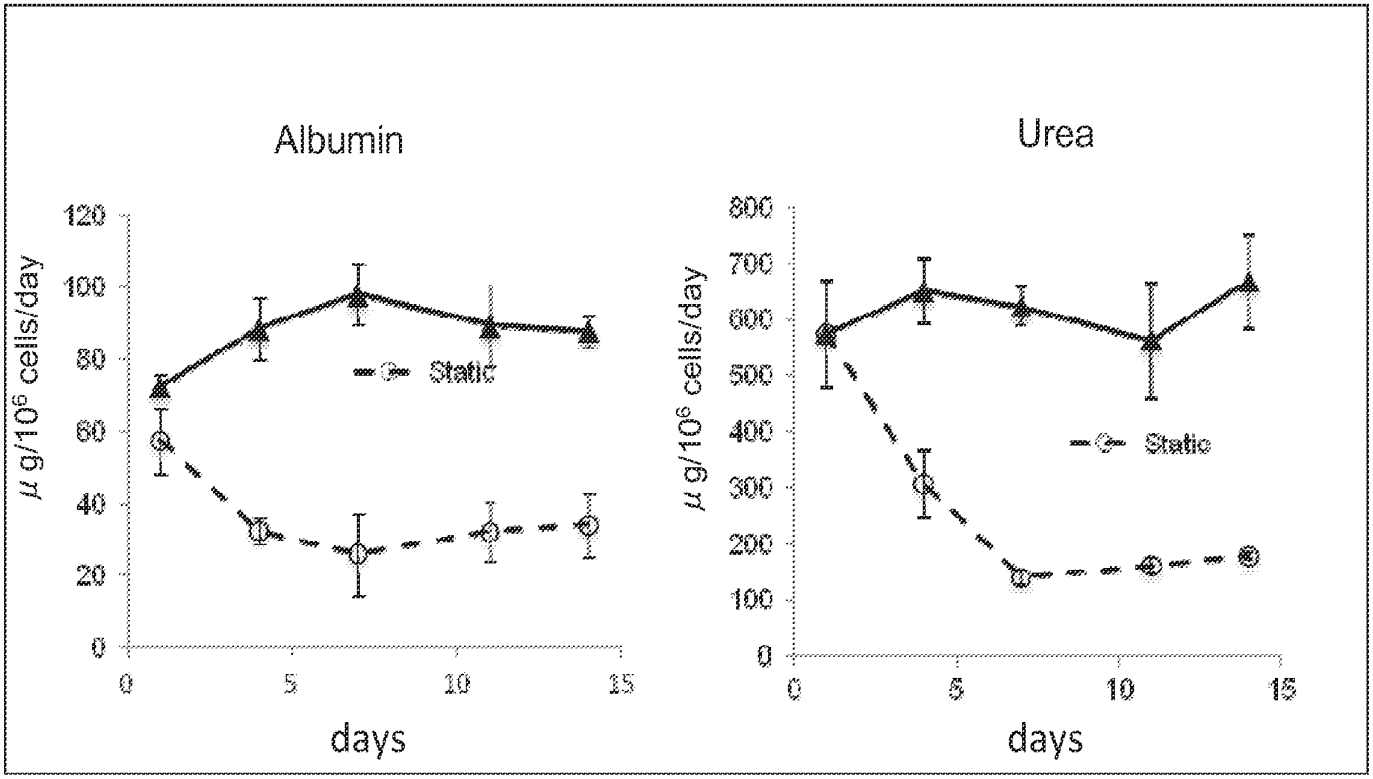


FIG. 23A

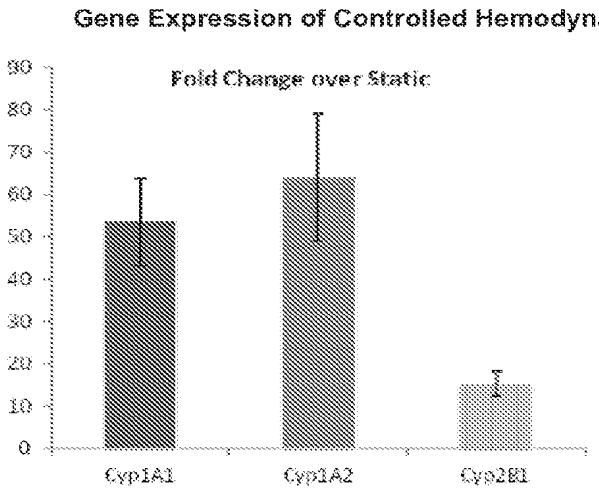


FIG. 23B

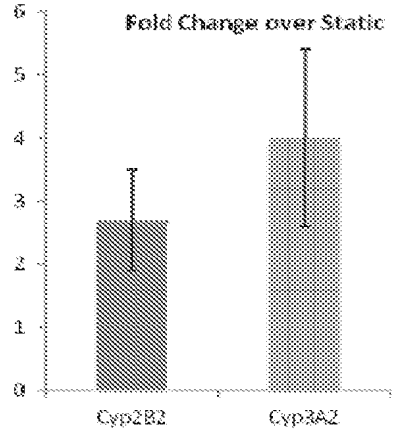


FIG. 23C

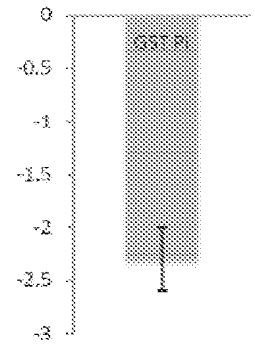
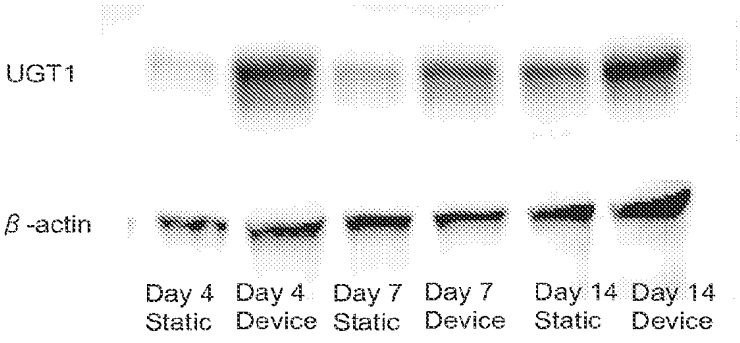


FIG. 23D



Effect of Controlled Hemodynamics in Device on UGT1 Protein Expression Over 2 Weeks

FIG. 24A

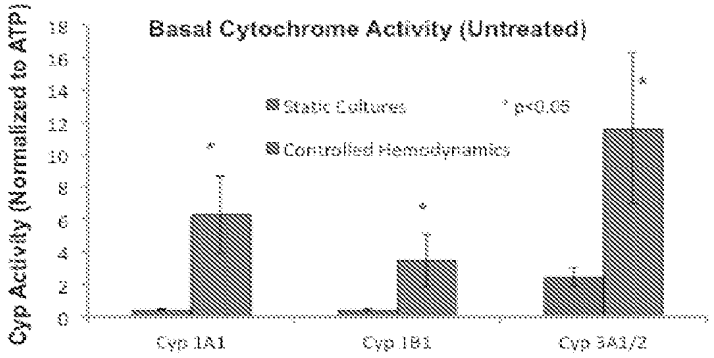
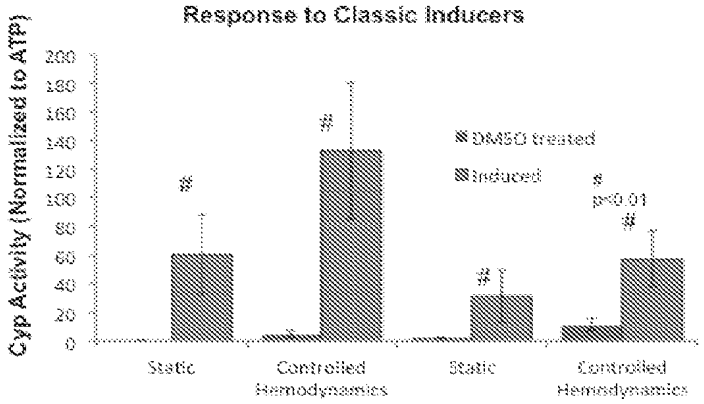
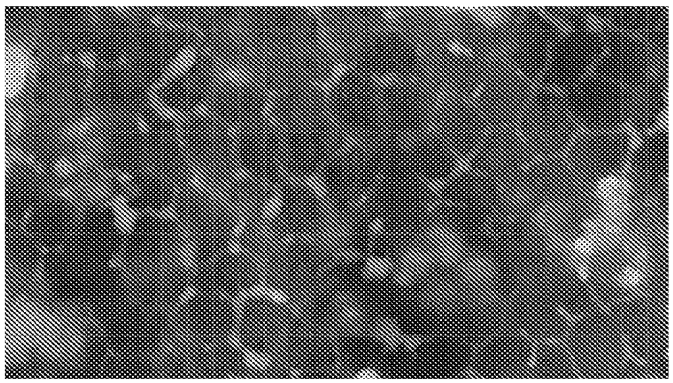


FIG. 24B



1A1/1B1	3A1/3A2
Inducer – 3 Methyl Cholanthrene Static – 1 µM Controlled Hemodynamics - 0.1 µM	Inducer – Dexamethasone Static – 50 µM Controlled Hemodynamics – 2.5 µM

FIG. 24C



Day 7 Controlled Hemodynamics:
Biliary Efflux Activity: CDFDA ➔ CDF

FIG. 25

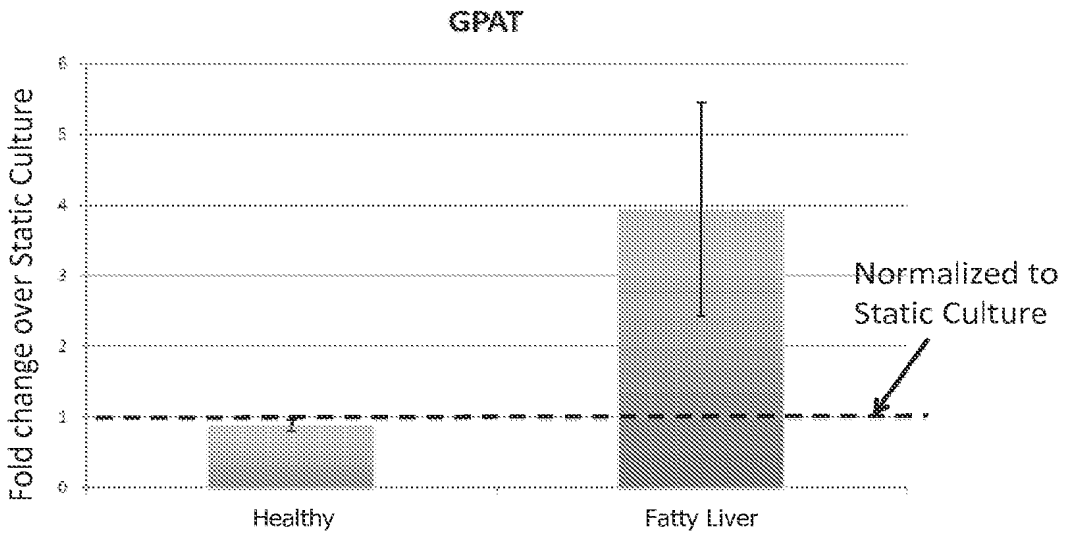

GPAT
Fold change over Static Culture
Normalized to Static Culture
Healthy
Fatty Liver

FIG. 26

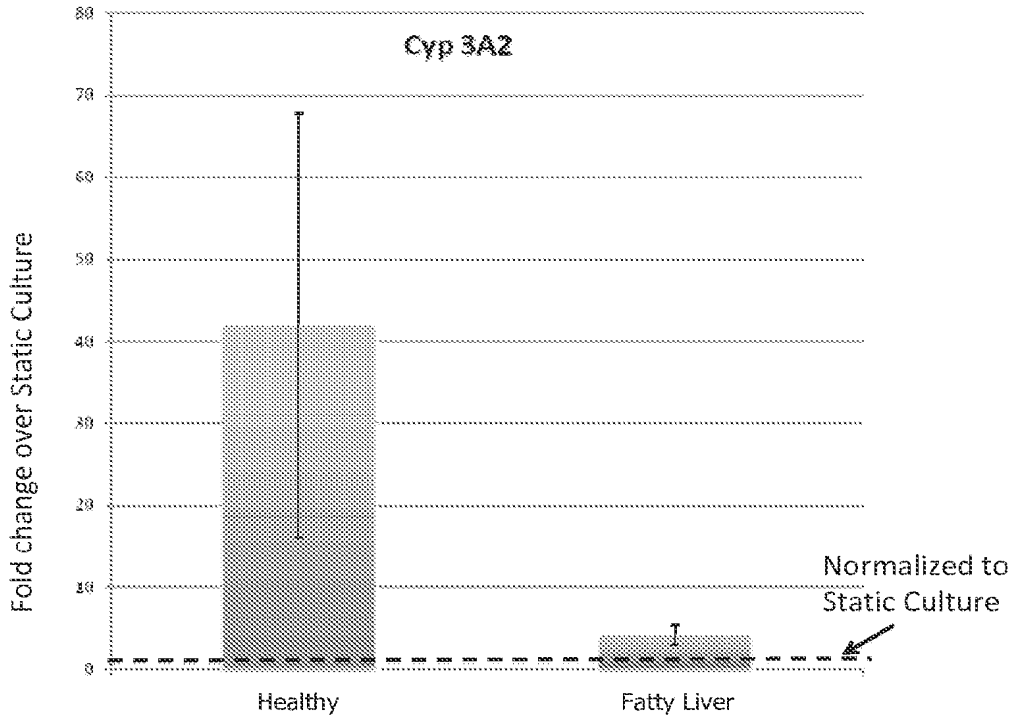

Cyp 3A2
Fold change over Static Culture
Normalized to Static Culture
Healthy
Fatty Liver

FIG. 27A

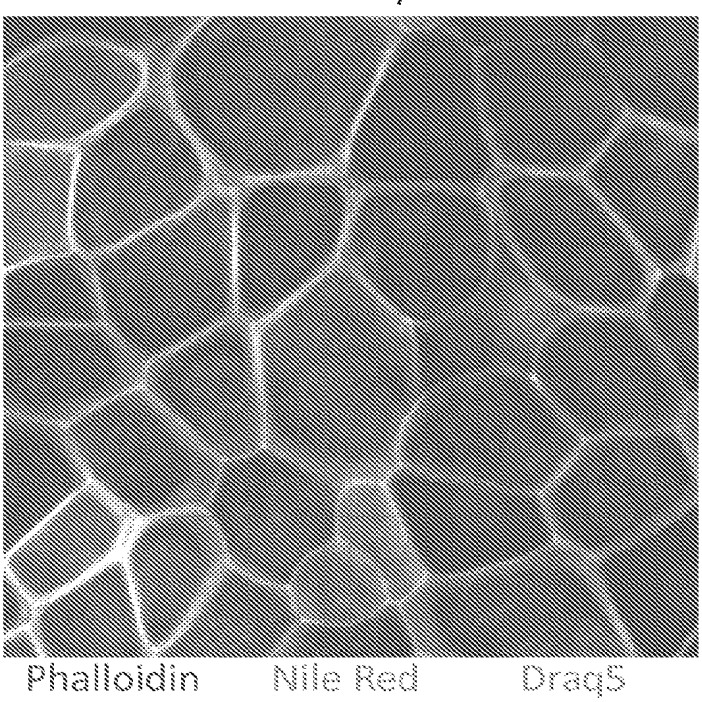


FIG. 27B

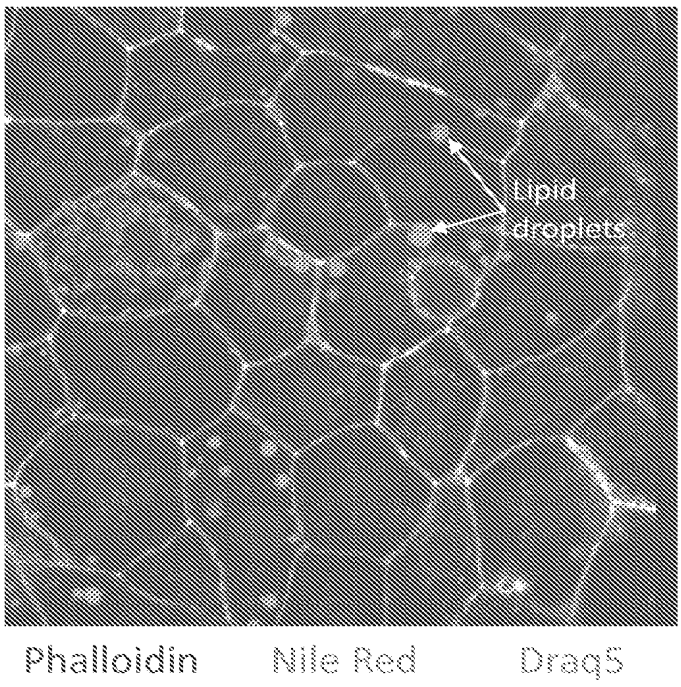
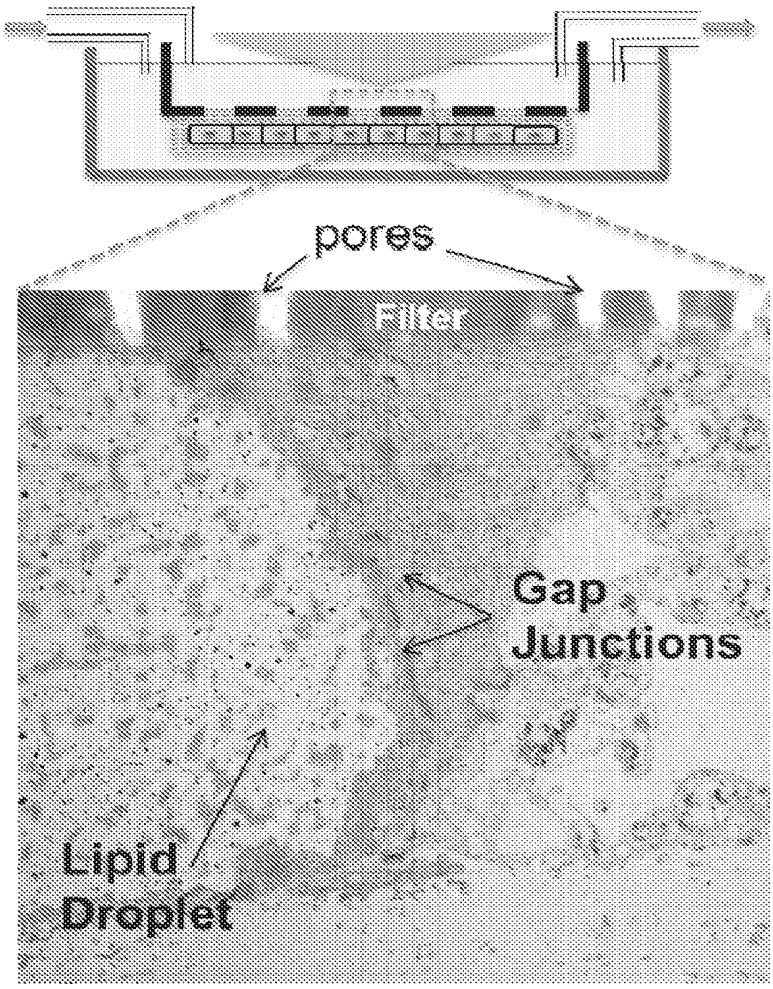


FIG. 28



17.5 mM Glucose 2 µMol Insulin

FIG. 29A

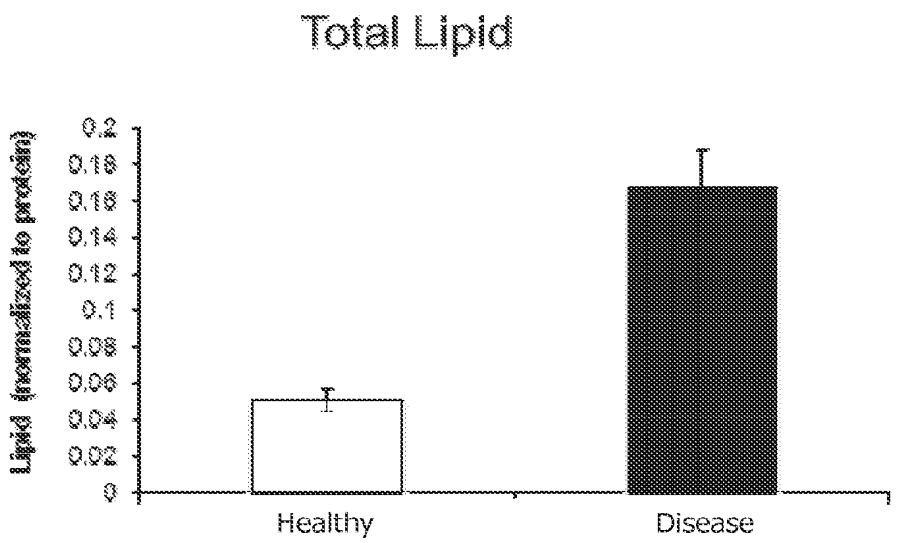


FIG. 29B

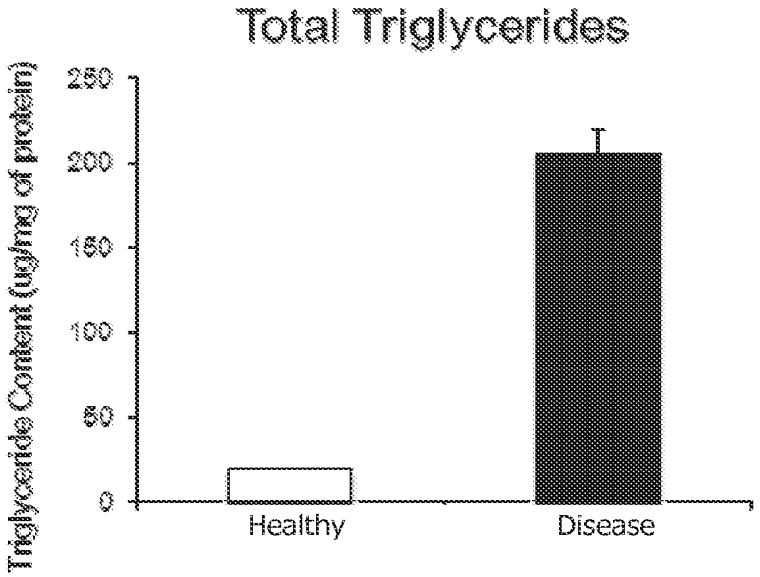


FIG. 30A

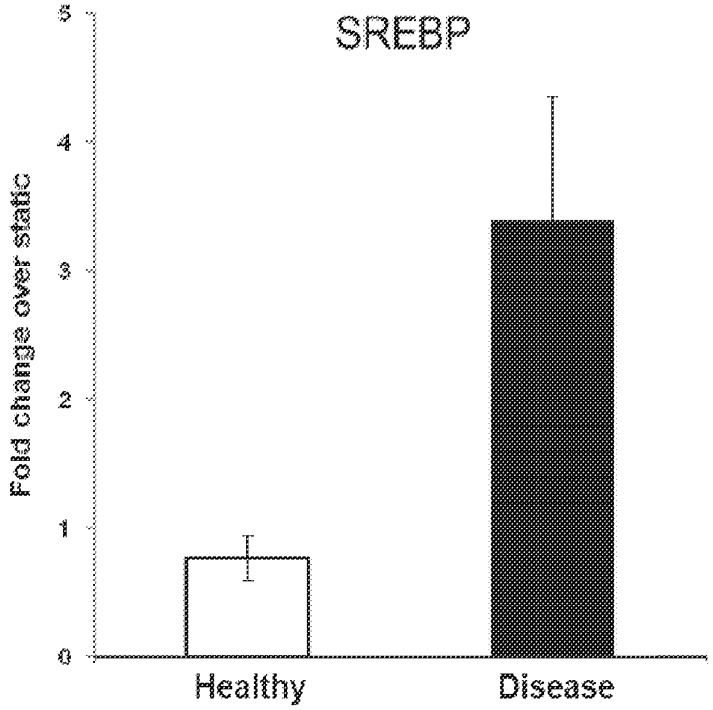


FIG. 30B

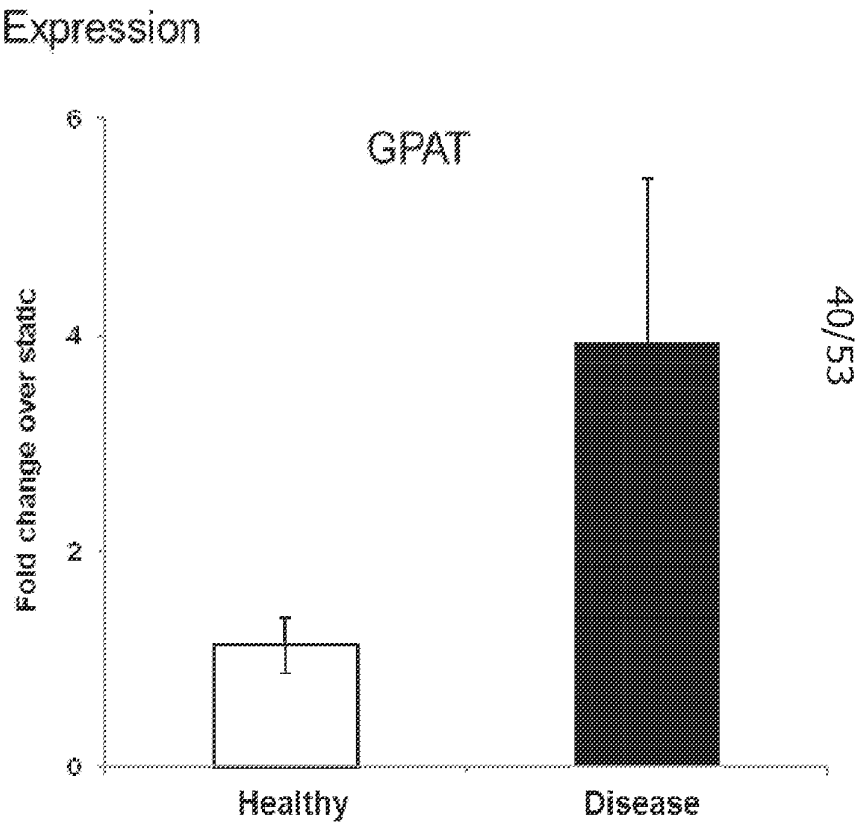


FIG. 31A

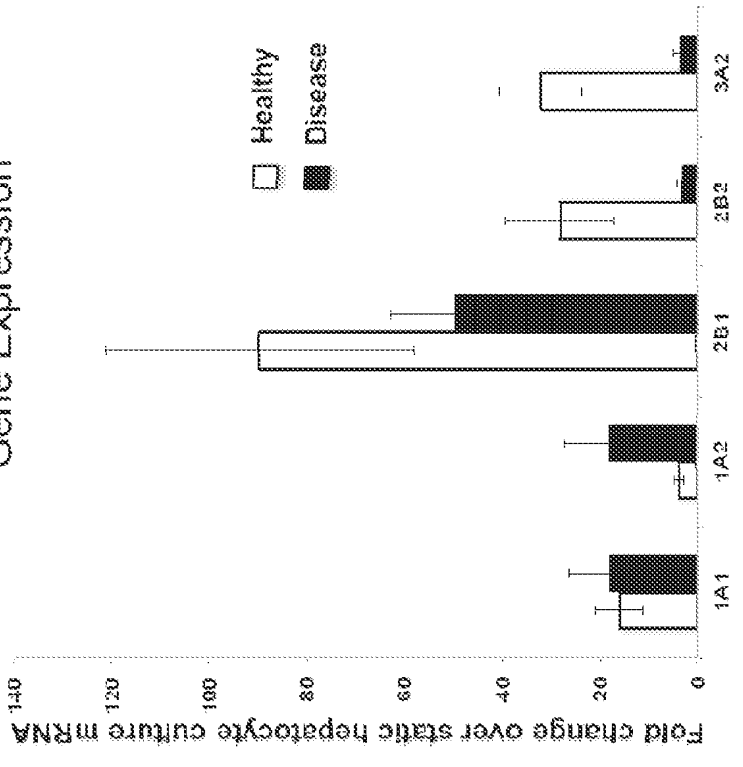


FIG. 31B

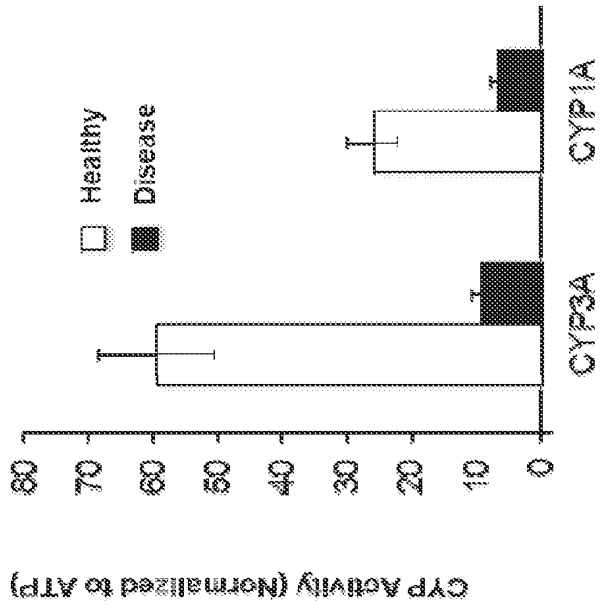


FIG. 32A

FIG. 32B

FIG. 32C

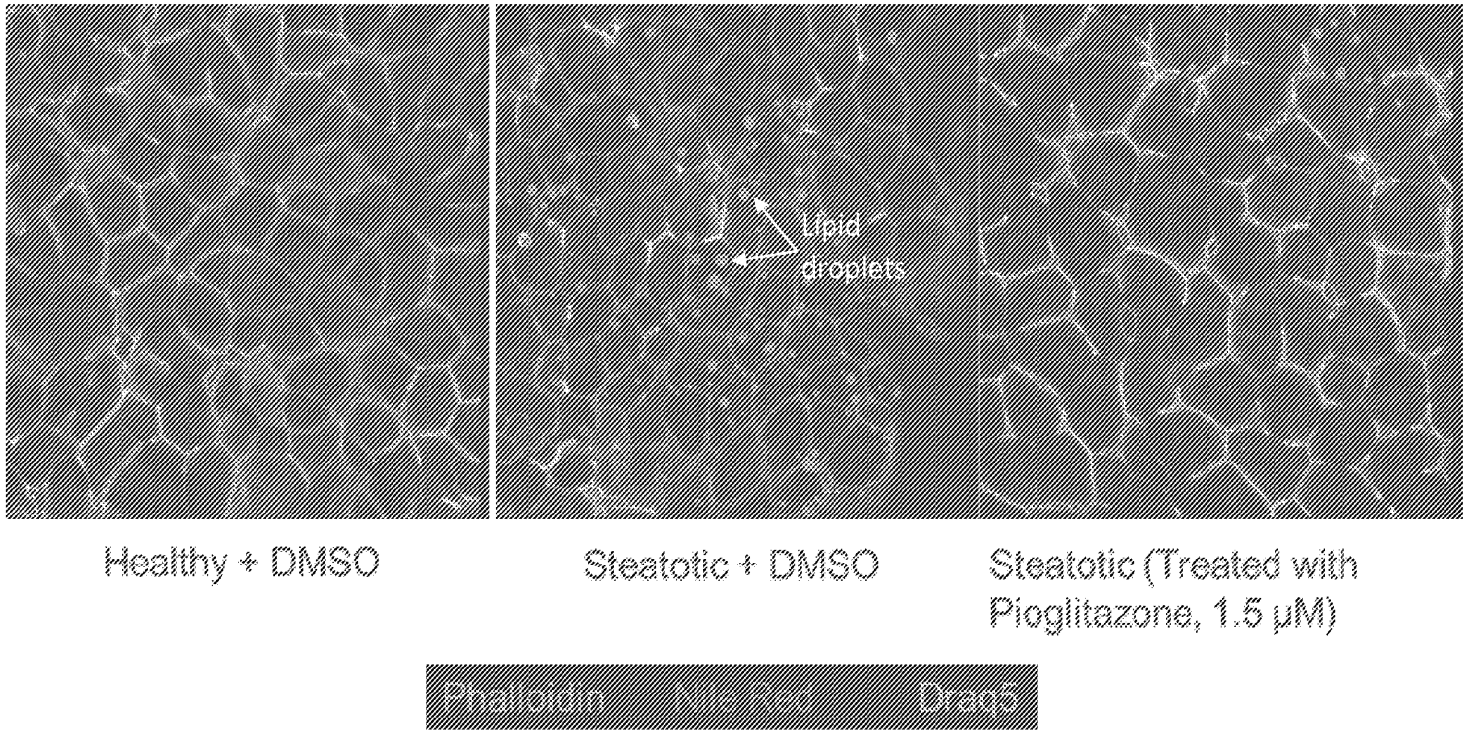


FIG. 33

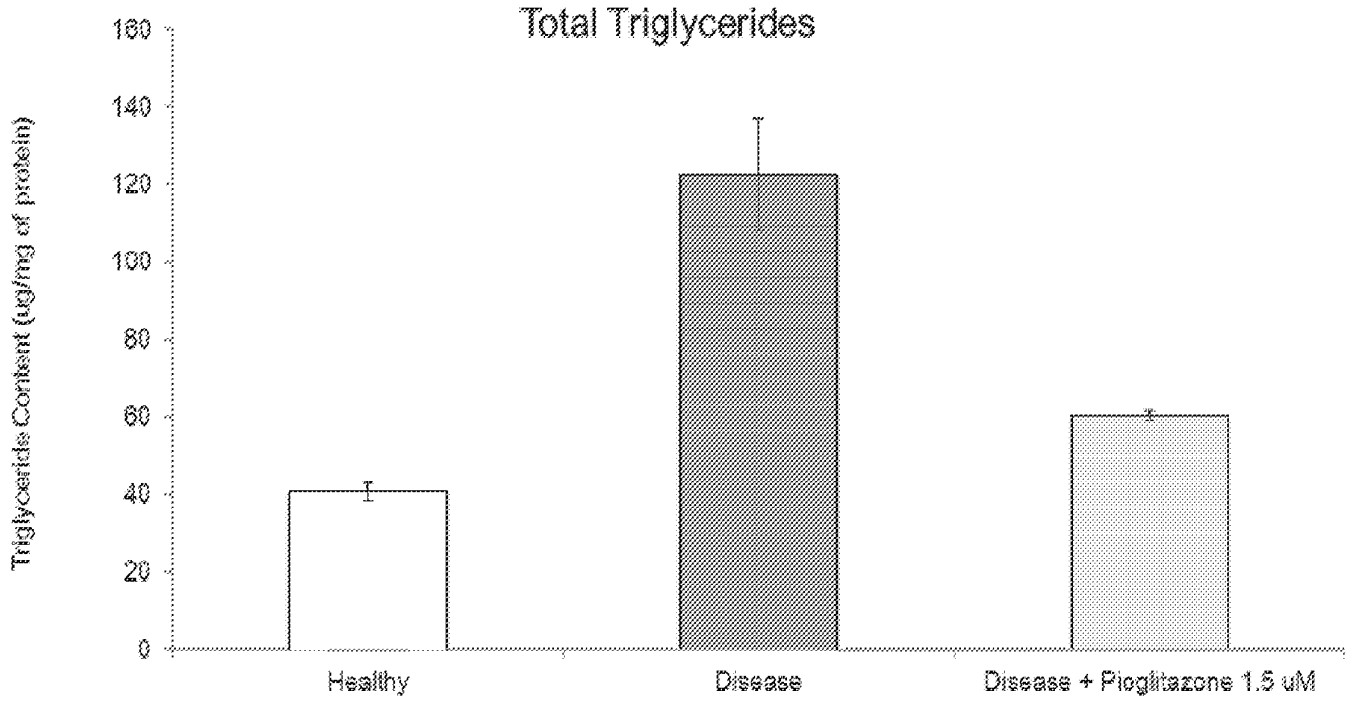

Total Triglycerides
Triglycerides Content (ug/mg of protein)
160
140
120
100
80
60
40
20
0
Healthy
Disease
Disease + Pioglitazone 1.5 uM

FIG. 34

FIG. 35A

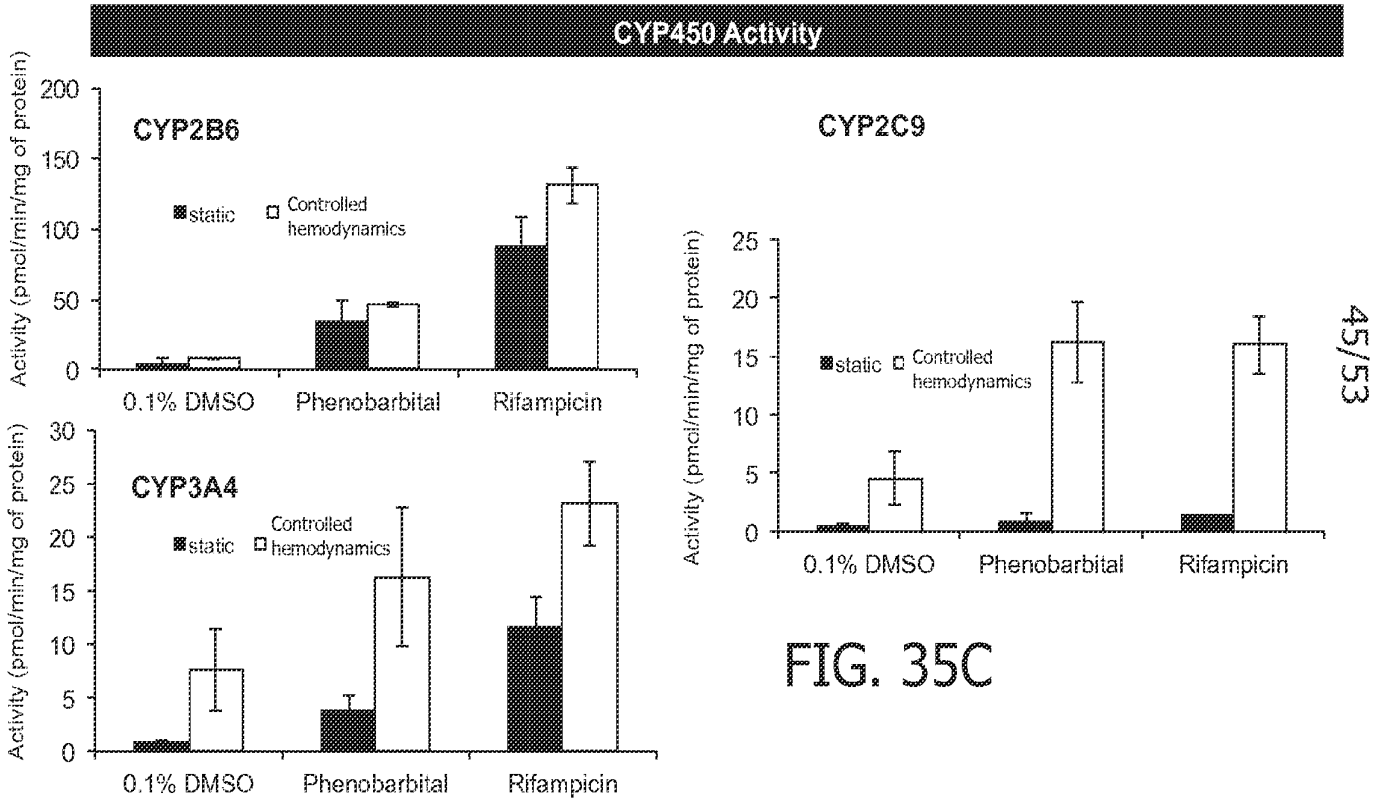


FIG. 35B

FIG. 35C

FIG. 36A

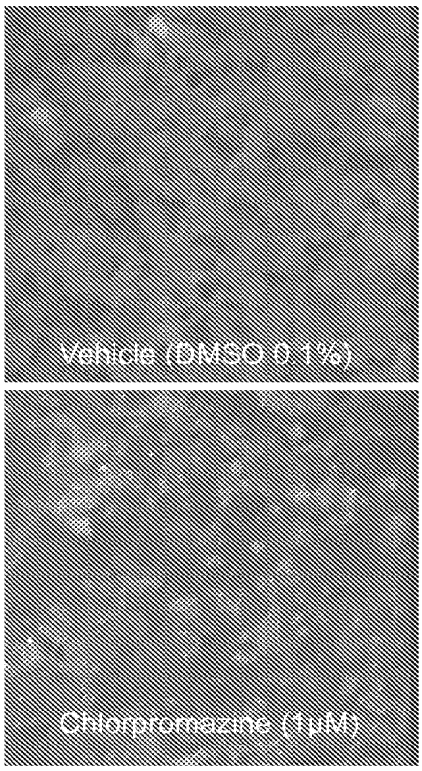
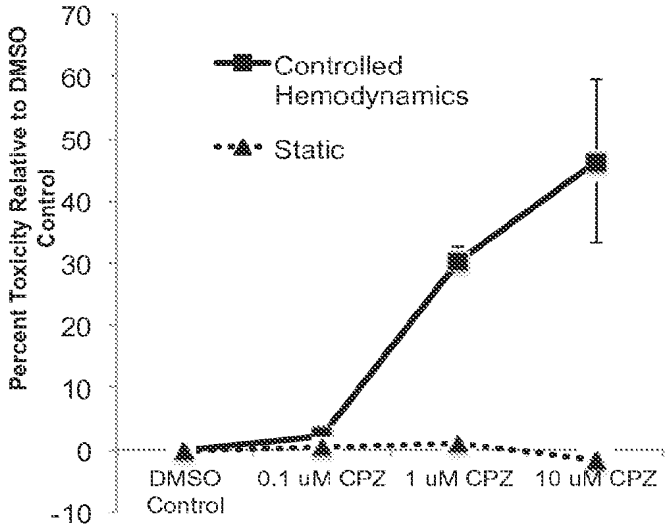


FIG. 36B



KalyCell Donor # N1309VT

FIG. 37A

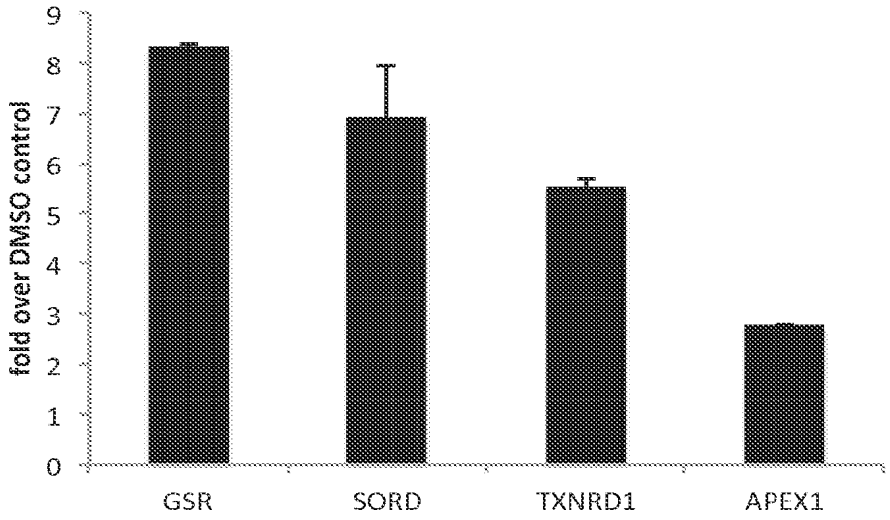


FIG. 37B

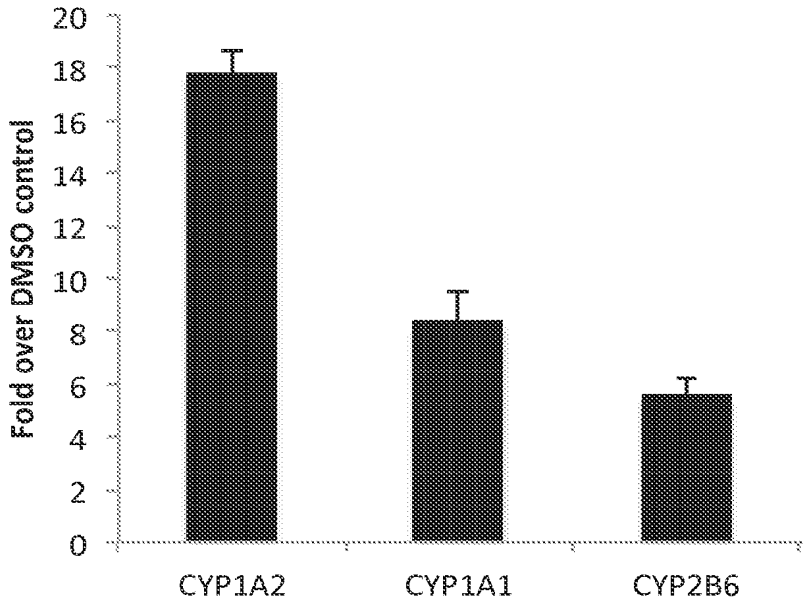


FIG. 38

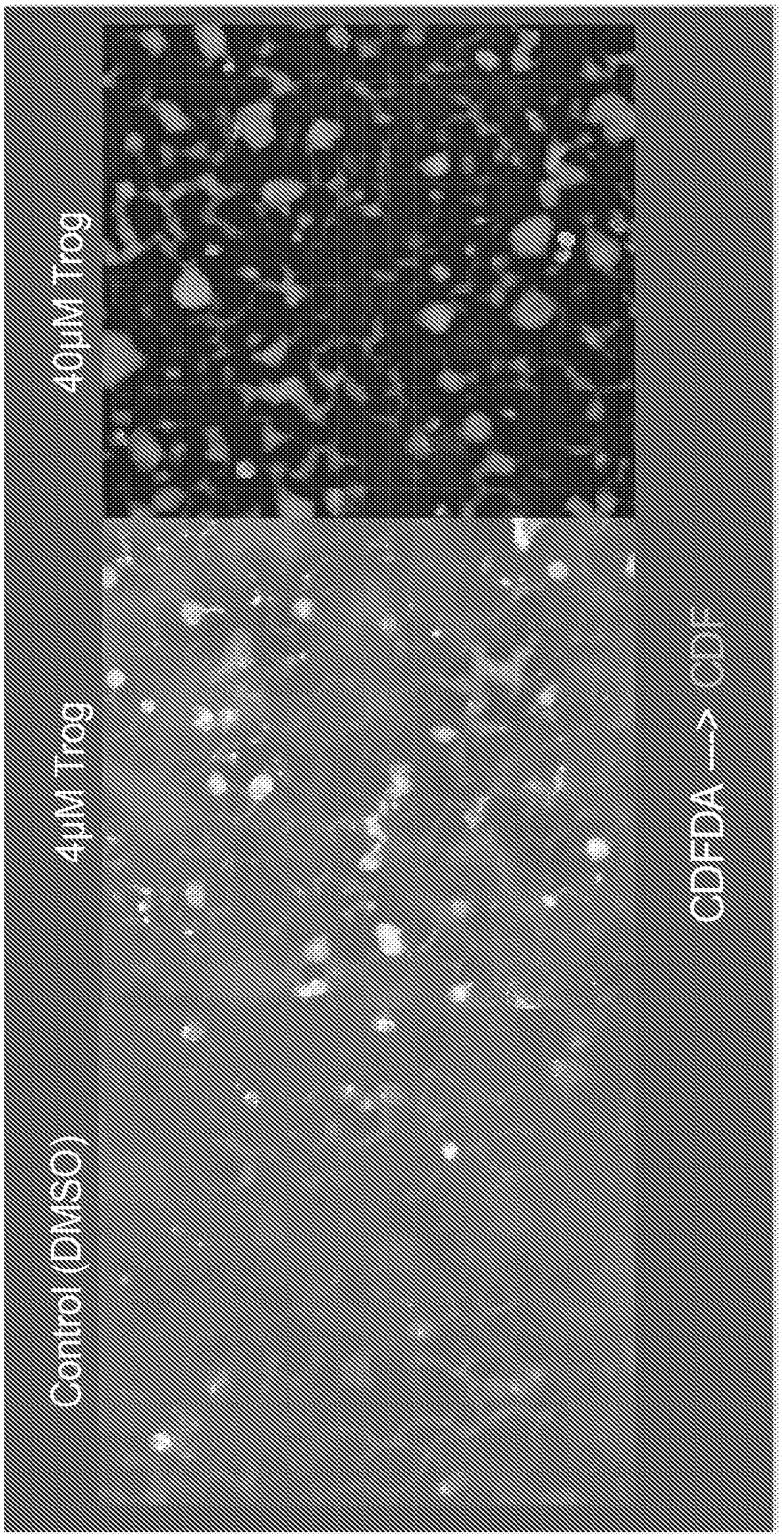


FIG. 39

FIG. 40

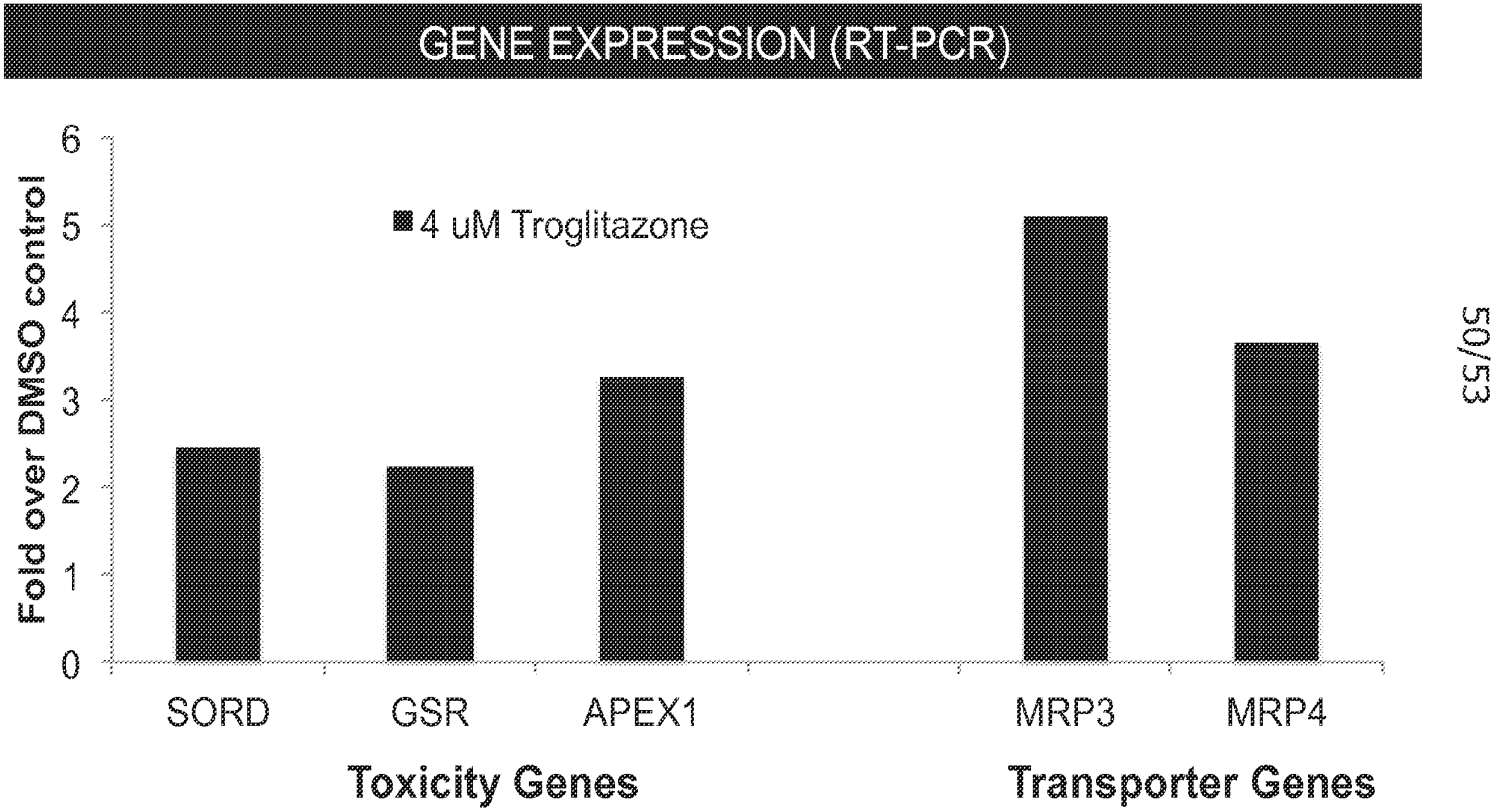

GENE EXPRESSION (RT-PCR)
Fold over DMSO control
0
1
2
3
4
5
6
4 uM Troglitazone
SORD
GSR
APEX1
MRP3
MRP4
Toxicity Genes
Transporter Genes

FIG. 41A

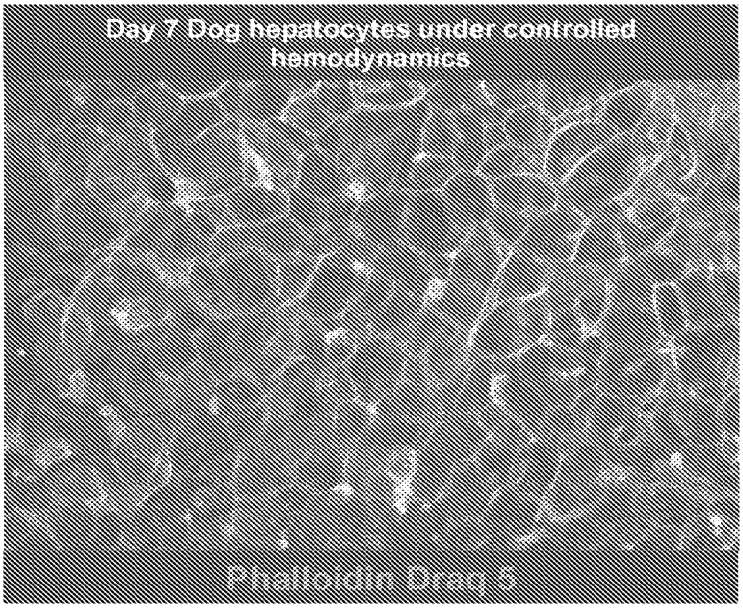


FIG. 41B

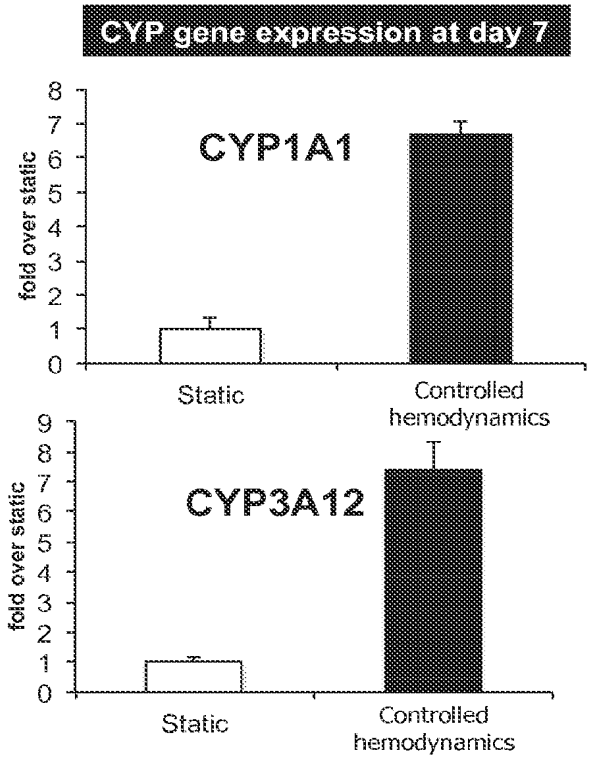


FIG. 42

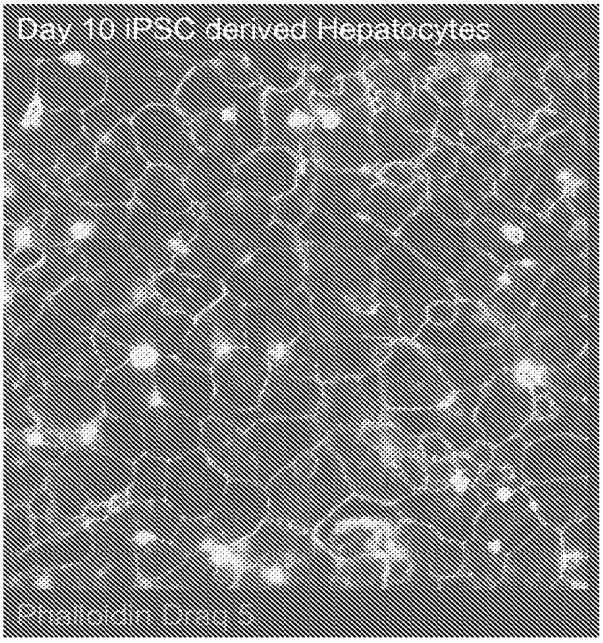


FIG. 43A

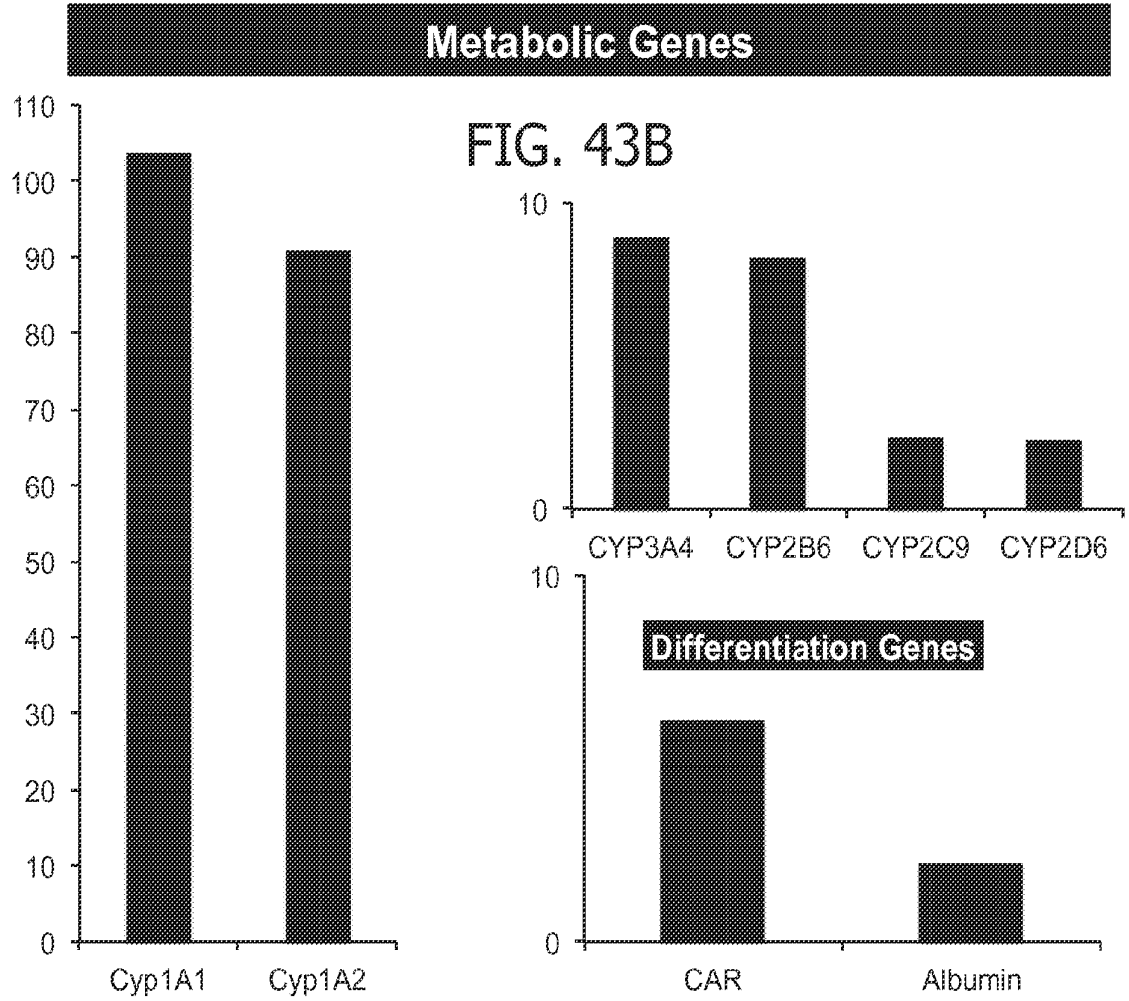


FIG. 43B

FIG. 43C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/061653

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/09(2010.01)i, C12M 3/00(2006.01)i, A01K 67/027(2006.01)i, G01N 33/15(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 5/09; C12Q 1/02; C12M 3/00; A01K 67/027; G01N 33/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:mimicking, tumor microenvironment, cell culture container, culture medium, tumor cell, shear stress, stromal cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 7811782 B2 (BLACKMAN, BRETT R. et al.) 12 October 2010 See abstract; claims 1-43; and figures 1-2.	1-7,16,18-20 ,148-151
Y	SMALLEY, KEIRAN SM et al., 'In vitro three-dimensional tumor microenvironment models for anticancer drug discovery', Expert Opinion on Drug Discovery, 2008, Vol.3, No.1, pp.1-10 See abstract; and page 7, right column.	1-7,16,18-20 ,148-151
A	SAGGAR, JASDEEP K. et al., 'The tumor microenvironment and strategies to improve drug distribution', Frontiers in Oncology, 10 June 2013, Vol.3, Article No.154, (pp.1-6) See the whole document.	1-7,16,18-20 ,148-151
A	QAZI, HENRY et al., 'Fluid shear stress regulates the invasive potential of glioma cells via modulation of migratory activity and matrix metalloproteinase expression', PLoS One, 2011, Vol.6, Issue No.5, Article No.e20348 (pp.1-13) See the whole document.	1-7,16,18-20 ,148-151

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
04 February 2015 (04.02.2015)	**04 February 2015 (04.02.2015)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TANNOCK, IAN F. et al., 'Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy', Clinical Cancer Research, 2002, Vol.8, No.3, pp.878-884 See the whole document.	1-7,16,18-20,148-151

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/061653

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: See Extra Sheet
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Extra Sheet.

3. ☒ Claims Nos.: See Extra Sheet
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

Continuation of: Box No. II.

2. Claims 10-14, 25-28, 30, 46-47, 49-50, 52-60, 63, 65, 67, 74, 78-81, 83-84, 90, 94, 97, 102-104, 106-107, 109, 111, 114-115, 118, 122-123, 129-130, 134-138, 143-147, 153, 156, 160, 162-163, 165-166, 168-175, 178, 180, 183, 187-188, 190-191, 193, 199, 202-203, 213, 219-221, 223

Claims 10-14, 25-28, 30, 46-47, 49-50, 52-60, 63, 65, 67, 74, 78-81, 83-84, 90, 94, 97, 102-104, 106-107, 109, 111, 114-115, 118, 122-123, 129-130, 134-138, 143-147, 153, 156, 160, 162-163, 165-166, 168-175, 178, 180, 183, 187-188, 190-191, 193, 199, 202-203, 213, 219-221 and 223 are unclear since they refer to multiple dependent claims which do not comply with PCT Rule 6.4(a).

Claim 26 refers to itself, thereby rendering the definition of the subject matter of said claim unclear (PCT Article 6).

3. Claims 8-9, 15, 17, 21-24, 29, 31-45, 48, 51, 61-62, 64, 66, 68-73, 75-77, 82, 85-89, 91-93, 95-96, 98-101, 105, 108, 110, 112-113, 116-117, 119-121, 124-128, 131-133, 139-142, 152, 154-155, 157-159, 161, 164, 167, 176-177, 179, 181-182, 184-186, 189, 192, 194-198, 200-201, 204-212, 214-218, 222

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/061653

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 7811782 B2	12/10/2010	CA 2675147 A1	26/03/2009
		CN 101680018 A	24/03/2010
		EP 2118268 A2	18/11/2009
		JP 2010-515458 A	13/05/2010
		JP 2012-080888 A	26/04/2012
		US 2009-0053752 A1	26/02/2009
		US 2011-0059480 A1	10/03/2011
		US 8871461 B2	28/10/2014
		WO 2009-038594 A2	26/03/2009
		WO 2009-038594 A3	30/12/2009

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International Bureau

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(71) Applicant: GENEX CORPORATION [US/US]; 16020 Industrial Drive, Gaithersburg, MD 20877 (US).

(72) Inventors: LADNER, Robert, Charles ; 3827 Green Valley, Iamsville, MD 21754 (US). BIRD, Robert, Earl ; 3903 Morrell Court, Kensington, MD 20895 (US).

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(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.

(54) Title: SINGLE POLYPEPTIDE CHAIN BINDING MOLECULES

(57) Abstract

A single polypeptide chain binding molecule which has binding specificity substantially similar to the binding specificity of the light and heavy chain aggregate variable region of an antibody, genetic sequences coding therefor, recombinant DNA methods of producing such molecule and uses for such molecule.

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SINGLE POLYPEPTIDE CHAIN BINDING MOLECULES

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of Application Serial No. 902,971, filed September 2, 1986, the contents of which are herein fully incorporated by reference.

Field of the Invention

The present invention relates to single polypeptide chain binding molecules having the three dimensional folding, and thus the binding ability and specificity, of the variable region of an antibody. Methods of producing these molecules by genetic engineering are also disclosed.

Description of the Background Art

The advent of modern molecular biology and immunology has brought about the possibility of producing large quantities of biologically active materials in highly reproduceable form and with low cost. Briefly, the gene sequence coding for a desired natural protein is isolated, replicated (cloned) and introduced into a foreign host such as a bacterium, a yeast (or other fungi) or a mammalian cell line in culture, with appropriate regulatory control signals. When the signals are activated, the gene is transcribed and translated, and expresses the desired protein. In this manner, such useful biologically active materials as hormones, enzymes or antibodies have been cloned and expressed in foreign hosts.

One of the problems with this approach is that it is limited by the "one gene, one polypeptide chain" principle of molecular biology. In other words, a genetic sequence codes for a single polypeptide chain. Many biologically active polypeptides, however, are aggregates of two or more chains. For example, antibodies are three-dimensional aggregates of two heavy and two light chains. In the same manner, large enzymes such as aspartate transcarbamylase, for example, are aggregates of six catalytic and six regulatory chains, these chains being different. In order to produce such complex materials by recombinant DNA technology in foreign hosts, it becomes necessary to clone and express a gene coding for each one of the different kinds of polypeptide chains. These genes can be expressed in separate hosts. The resulting polypeptide chains from each host would then have to be reaggregated and allowed to refold together in solution. Alternatively, the two or more genes coding for the two or more polypeptide chains of the aggregate could be expressed in the same host simultaneously, so that refolding and reassociation into the native structure with biological activity will occur after expression. The approach, however, necessitates expression of multiple genes, and as indicated, in some cases, in multiple and different hosts. These approaches have proved to be inefficient.

Even if the two or more genes are expressed in the same organism it is quite difficult to get them all expressed in the required amounts.

A classical example of multigene expression to form multimeric polypeptides is the expression by recombinant DNA technology of antibodies. Genes for heavy and light chains have been introduced into ap-

propriate hosts and expressed, followed by reaggregation of these individual chains into functional antibody molecules (see for example Munro, Nature, 312:597 (1984); Morrison, S.L. Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986)); Wood et al., Nature, 314: 446-449 (1985)).

Antibody molecules have two generally recognized regions, in each of the heavy and light chains. These regions are the so-called "variable" region which is responsible for binding to the specific antigen in question, and the so-called "constant" region which is responsible for biological effector responses such as complement binding, etc. The constant regions are not necessary for antigen binding. The constant regions have been separated from the antibody molecule, and biologically active (i.e. binding) variable regions have been obtained.

The variable regions of an antibody are composed of a light chain and a heavy chain. Light and heavy chain variable regions have been cloned and expressed in foreign hosts, and maintain their binding ability (Moore et al, European Patent Publication 0088994 (published September 21, 1983)).

Further, it is by now well established that all antibodies of a certain class and their Fab fragments whose structures have been determined by X-ray crystallography, even when from different species, show closely similar variable regions despite large differences in the hypervariable segments. The immunoglobulin variable region seems to be tolerant toward mutations in the combining loops. Therefore, other than in the hypervariable regions, most of the so

called "variable" regions of antibodies, which are defined by both heavy and light chains, are in fact quite constant in their three dimensional arrangement. See, for example, Huber, R., "Structural Basis for Antigen-Antibody Recognition," Science, 233:702-703 (1986).

It would be very efficient if one could produce single polypeptide-chain molecules which have the same biological activity as the multiple chain aggregates such as, for example, multiple chain antibody aggregates or enzyme aggregates. Given the "one gene-one-polypeptide chain" principle, such single chain molecules would be more readily produceable, and would not necessitate multiple hosts or multiple genes in the cloning and expression. In order to accomplish this, it is first necessary to devise a method for generating single chain structures from two-chain aggregate structures, wherein the single chain will retain the three-dimensional folding of the separate natural aggregate of two polypeptide chains.

While the art has discussed the study of proteins in three dimensions, and has suggested modifying their architecture (see, for example, the article "Protein Architecture: Designing from the Ground Up," by Van Brunt, J., BioTechnology, 4: 277-283 (April, 1986)), the problem of generating single chain structures from multiple chain structures, wherein the single chain structure will retain the three-dimensional architecture of the multiple chain aggregate, has not been satisfactorily addressed.

Given that methods for the preparation of genetic sequences, their replication, their linking to expres-

sion control regions, formation of vectors therewith and transformation of appropriate hosts are well understood techniques, it would indeed be greatly advantageous to be able to produce, by genetic engineering, single polypeptide chain binding proteins having the characteristics and binding ability of multi chain variable regions of antibody molecules.

SUMMARY OF THE INVENTION

The present invention starts with a computer based system and method to determine chemical structures for converting two naturally aggregated but chemically separated light and heavy polypeptide chains from an antibody variable region into a single polypeptide chain which will fold into a three dimensional structure very similar to the original structure made of the two polypeptide chains.

The single polypeptide chain obtained from this method can then be used to prepare a genetic sequence coding therefor. The genetic sequence can then be replicated in appropriate hosts, further linked to control regions, and transformed into expression hosts, wherein it can be expressed. The resulting single polypeptide chain binding protein, upon refolding, has the binding characteristics of the aggregate of the original two (heavy and light) polypeptide chains of the variable region of the antibody.

The invention therefore comprises:

A single polypeptide chain binding molecule which has binding specificity substantially similar to the binding specificity of the light and heavy chain aggregate variable region of an antibody.

The invention also comprises genetic sequences coding for the above mentioned single polypeptide chain, cloning and expression vectors containing such genetic sequences, hosts transformed with such vectors, and methods of production of such polypeptides by expression of the underlying genetic sequences in such hosts.

The invention also extends to uses for the binding proteins, including uses in diagnostics, therapy, in vivo and in vitro imaging, purifications, and biosensors. The invention also extends to the single chain binding molecules in immobilized form, or in detectably labelled forms for utilization in the above mentioned diagnostic, imaging, purification or biosensor applications. It also extends to conjugates of the single polypeptide chain binding molecules with therapuetic agents such as drugs or specific toxins, for delivery to a specific site in an animal, such as a human patient.

Essentially all of the uses that the prior art has envisioned for monoclonal or polyclonal antibodies, or for variable region fragments thereof, can be considered for the molecules of the present invention.

The advantages of single chain over conventional antibodies are smaller size, greater stability and significantly reduced cost. The smaller size of single chain antibodies may reduce the body's immunologic reaction and thus increase the safety and efficacy of therapeutic applications. Conversely, the single chain antibodies could be engineered to be highly antigenic. The increased stability and lower cost permits greater use in biosensors and protein purifica-

tion systems. Because it is a smaller and simpler protein, the single chain antibody is easier to further modify by protein engineering so as to improve both its binding affinity and its specificity. Improved affinity will increase the sensitivity of diagnosis and detection and detection systems while improved specificity will reduce the number of false positives observed.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention as defined in the claims can be better understood with reference to the text and to the following drawings, as follows:

Figure 1 is a block diagram of the hardware aspects of the serial processor mode of the present invention.

Figure 2 is a block diagram of an alternate embodiment of the hardware aspects of the present invention.

Figure 3 is a block diagram of the three general steps of the present invention.

Figure 4 is a block diagram of the steps in the site selection step in the single linker embodiment.

Figure 5A is a schematic two dimensional simplified representation of the light chain L and heavy chain H of two naturally aggregated antibody variable region F_v polypeptide chains used to illustrate the site selection process.

Figure 5B is a two dimensional representation of the three dimensional relationship of the two aggregated polypeptide chains showing the light chain L (----) and the heavy chain H (-) of the variable region of one antibody.

Figure 6A is a simplified two dimensional schematic diagram of the two polypeptide chains showing the location of the residue Tau 1 and the residue Sigma 1.

Figure 6B is a two dimensional representation of the actual relationship of the two polypeptide chains showing the residue Tau 1 and the residue Sigma 1.

Figure 7 shows in very simplified schematic way the concept of the direction linkers that are possible between the various possible sites on the light chain L and the heavy chain H in the residue Tau 1 and residue Sigma 1 respectively.

Figure 8A is a two dimensional simplified schematic diagram of a single chain antibody linking together two separate chains ((Heavy) and (light)) by linker 1 (----) to produce a single chain antibody.

Figure 8B is a two dimensional representation showing a single chain antibody produced by linking two aggregated polypeptide chains using linker 1.

Figure 9 shows a block diagram of candidate selection for correct span.

Figure 10 shows a block diagram of candidate selection for correct direction from N terminal to C terminal.

Figure 11 shows a comparison of direction of a gap to direction of a candidate.

Figure 12 shows a block diagram of candidate selection for correct orientation at both ends.

Figure 13 shows a block diagram of selection of sites for the two-linker embodiment.

Figure 14 shows examples of rules by which candidates may be ranked.

Figure 15A shows a two-dimensional simplified representation of the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the first two sites to be linked.

Figure 15B shows a two-dimensional representation of the three-dimensional relationships between the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the regions in which the second sites to be linked can be found and the linker between the first pair of sites.

Figure 16A shows the two-dimensional simplified representation of the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the regions in which the second sites to be linked can be found and the linker between the first pair of sites.

Figure 16B shows the two-dimensional representation of the three-dimensional relationships between the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the regions in which the second sites to be linked can be found and the linker between the first pair of sites.

Figure 17A shows the two-dimensional simplified representation of the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the second linker and the portions of the native protein which are lost.

Figure 17B shows the two-dimensional representation of the three-dimensional relationships between the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the second linker and the portions of native protein which are lost.

Figure 18 shows the two-dimensional simplified representation of the variable domain of an Fv light chain, L, and the variable domain of an Fv heavy chain, H, showing the complete construction.

Figure 19 shows a block diagram of the parallel processing mode of the present invention.

Figure 20A shows five pieces of molecular structure. The uppermost segment consists of two peptides joined by a long line. The separation between the peptides is 12.7 A. The first C of each peptide lies on the X-axis. The two dots indicate the standard reference point in each peptide.

Below the gap are four linker candidates (labeled 1,2,3 & 4), represented by a line joining the alpha carbons. In all cases, the first and penultimate alpha carbons are on lines parallel to the X-axis, spaced 8.0 A apart. Note that the space between dots in linker 1 is much shorter than in the gap.

Figure 20B shows the initial peptides of linkers 2, 3, and 4 which have been aligned with the first peptide of the gap. For clarity, the linkers have been translated vertically to their original positions.

The vector from the first peptide in the gap to the second peptide in the gap lies along the X-axis, a corresponding vector for linkers 3 and 4 also lies along the X-axis. Linker 2, however, has this vector pointing up and to the right, thus linker 2 is rejected.

Figure 20C shows the ten atoms which compose the initial and final peptides of linkers 3 and 4, which have been least-squares fit to the corresponding atoms from the gap. These peptides have been drawn in.

Note that in the gap and in linker 4 the final peptide points down and lies more-or-less in the plane of the paper. In linker 3, however, this final peptide points down and to the left and is twisted about 90 degrees so that the carbonyl oxygen points toward the viewer. Thus linker 3 is rejected.

Sections B and C are stereo diagrams which may be viewed with the standard stereo viewer provided.

Figure 21 shows the nucleotide sequence and translation of the sequence for the heavy chain of a mouse anti bovine growth hormone (BGH) monoclonal antibody.

Figure 22 shows the nucleotide sequence and translation of the sequence for the light chain of the same monoclonal antibody as that shown in Figure 21.

Figure 23 is a plasmid restriction map containing the variable heavy chain sequence (pGX3772) and that containing the variable light sequence (pGX3773) shown in figures 21 and 22.

Figure 24 shows construction TRY40 comprising the nucleotide sequence and its translation sequence of a single polypeptide chain binding protein prepared according to the methods of the invention.

Figure 25 shows a restriction map of the expression vector pGX3776 carrying a single chain binding protein, the sequence of which is shown in Figure 24. In this and subsequent plasmid maps (Figures 27 and 29) the hashed bar represents the promoter O_L/P_R sequence and the solid bar represents heavy chain variable region sequences.

Figure 26 shows the sequences of TRY61, another single chain binding protein of the invention.

Figure 27 shows expression plasmid pGX4904 carrying the genetic sequence shown in Figure 26.

Figure 28 shows the sequences of TRY59, another single chain binding protein of the invention.

Figure 29 shows the expression plasmid pGX 4908 carrying the genetic sequence shown in Figure 28.

Figures 30A, 30B, 30C, and 30D (stereo) are explained in detail in Example 1. They show the design and construction of double linked single chain antibody TRY40.

Figures 31A and 31B (stereo) are explained in detail in Example 2. They show the design and construction of single linked single chain antibody TRY61.

Figures 32A and 32B (stereo) are explained in detail in Example 3. They show the design and construction of single linked single chain antibody TRY59.

Figure 33 is explained in Example 4 and shows the sequence of TRY104b.

Figure 34 shows a restriction map of the expression vector pGX4910 carrying a single linker construction, the sequence of which is shown in Figure 33.

Figure 35 shows the assay results for BGH binding activity wherein strip one represents TRY61 and strip two represents TRY40.

Figure 36 is explained in Example 4 and shows the results of competing the F_{ab} portion of 3C2 monoclonal with TRY59 protein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

TABLE OF CONTENTS

II. Hardware and Software Environment

III. Single Linker Embodiment
- A. Plausible Site Selection
- B. Selection of Candidates
  - 1. Selecting Candidates with Proper Distance Between the N Terminal and the C Terminal.
  - 2. Selecting Candidates with Proper Direction From the N Terminal and the C Terminal.
  - 3. Selecting Candidates With Proper Orientation between the Termini.
- C. Ranking and Eliminating Candidates

IV. Double and Multiple Linker Embodiments
- A. Plausible Site Selection
- B. Candidate Selection and Candidate Rejection Steps

V. Parallel Processing Embodiment

VI. Preparation and Expression of Genetic Sequences and Uses

I. General Overview

The present invention starts with a computer based system and method for determining and displaying possible chemical structures (linkers) for converting two naturally aggregated but chemically separate heavy and light (H and L) polypeptide chains from the variable region of a given antibody into a single polypeptide chain which will fold into a three dimensional struc-

ture very similar to the original structure made of two polypeptide chains. The original structure is referred to hereafter as "native protein."

The first general step of the three general design steps of the present invention involves selection of plausible sites to be linked. In the case of a single linker, criteria are utilized to select a plausible site on each of the two polypeptide chains (H and L in the variable region) which will result in 1) a minimum loss of residues from the native protein chains and 2) a linker of minimum number of amino acids consistent with the need for stability. A pair of sites defines a gap to be bridged or linked.

A two-or-more-linker approach is adopted when a single linker can not achieve the two stated goals. In both the single-linker case and the two-or-more-linker case, more than one gap may be selected for use in the second general step.

The second general step of the present invention involves examining a data base to determine possible linkers to fill the plausible gaps selected in the first general step, so that candidates can be enrolled for the third general step. Specifically, a data base contains a large number of amino acid sequences for which the three-dimensional structure is known. In the second general step, this data base is examined to find which amino acid sequences can bridge the gap or gaps to create a plausible one-polypeptide structure which retains most of the three dimensional features of the native (i.e. original aggregate) variable region molecule. The testing of each possible linker proceeds in three general substeps. The first general substep utilizes the length of the possible candidate.

Specifically, the span or length (a scalar quantity) of the candidate is compared to the span of each of the gaps. If the difference between the length of the candidate and the span of any one of the gaps is less than a selected quantity, then the present invention proceeds to the second general substep with respect to this candidate. Figure 20A shows one gap and four possible linkers. The first linker fails the first general substep because its span is quite different from the span of the gap.

In the second general substep, called the direction substep, the initial peptide of the candidate is aligned with the initial peptide of each gap. Specifically, a selected number of atoms in the initial peptide of the candidate are rotated and translated as a rigid body to best fit the corresponding atoms in the initial peptide of each gap. The three dimensional vector (called the direction of the linker) from the initial peptide of the candidate linker to the final peptide of the candidate linker is compared to the three dimensional vector (call the direction of the gap) from the initial peptide of each gap to the final peptide of the same gap. If the ends of these two vectors come within a preselected distance of each other, the present invention proceeds to the third general substep of the second general step with respect to this candidate linker.

Figure 20B shows one gap and three linkers. All the linkers have the correct span and the initial peptides have been aligned. The second linker fails the second general substep because its direction is quite different from that of the gap; the other two linkers are carried forward to the third general substep of the second general step.

In the third general substep of the second design of the step of the present invention, the orientations of the terminal peptides of each linker are compared to the orientations of the terminal peptides of each gap. Specifically, a selected number of atoms (3, 4, or 5, 5 in the prefered embodiment) from the initial peptide of the candidate plus the same selected number of atoms (3, 4, or 5; 5 in the prefered embodiment) from the final peptide of the candidate are taken as a rigid body. The corresponding atoms from one of the gaps (viz 5 from the initial peptide and 5 from the final peptide) are taken as a second rigid body. These two rigid bodies are superimposed by a least-squares fit. If the error for this fit is below some preselected value, then the candidate passes the third general substep of the second general step and is enrolled for the third general step of the present invention. If the error is greater than or equal to the preselected value, the next gap is tested. When all gaps have been tested without finding a sufficiently good fit, the candidate is abandoned.

The third general step of the present invention results in the ranking of the linker candidates from most plausible to least plausible. The most plausible candidate is the fragment that can bridge the two plausible sites of one of the gaps to form a single polypeptide chain, where the bridge will least distort the resulting three dimensional folding of the single polypeptide chain from the natural folding of the aggregate of the two originally chemically separate chains.

In this third general step of the present invention, an expert operator uses an interactive computer-graphics approach to rank the linker candidates from

most plausible to least plausible. This ranking is done by observing the interactions between the linker candidate with all retained portions of the native protein. A set of rules are used for the ranking. These expert system rules can be built into the system so that the linkers are displayed only after they have satisfied the expert system rules that are utilized.

The present invention can be programmed so that certain expert rules are utilized as a first general substep in the third general step to rank candidates and even eliminate unsuitable candidates before visual inspection by an expert operator, which would be the second general substep of the third general step. These expert rules assist the expert operator in ranking the candidates from most plausible to least plausible. These expert rules can be modified based on experimental data on linkers produced by the system and methods of the present invention.

The most plausible candidate is a genetically producible single polypeptide chain binding molecule which has a very significantly higher probability (a million or more as compared to a random selection) of folding into a three dimensional structure very similar to the original structure made of the heavy and light chains of the antibody variable region than would be produced if random selection of the linker was done. In this way, the computer based system and method of the present invention can be utilized to engineer single polypeptide chains by using one or more linkers which convert naturally aggregated but chemically separated polypeptide chains into the desired single chain.

The elected candidate offers to the user a linked chain structure having a very significantly increased

probability of proper folding than would be obtained using a random selection process. This means that the genetic engineering aspect of creating the desired single polypeptide chain is significantly reduced, since the number of candidates that have to be genetically engineered in practice is reduced by a corresponding amount. The most plausible candidate can be used to genetically engineer an actual molecule.

The parameters of the various candidates can be stored for later use. They can also be provided to the user either visually or recorded on a suitable media (paper, magnetic tape, color slides, etc.). The results of the various steps utilized in the design process can also be stored for later use or examination.

The design steps of the present invention operate on a conventional minicomputer system having storage devices capable of storing the amino acid sequence-structure data base, the various application programs utilized and the parameters of the possible linker candidates that are being evaluated.

The minicomputer CPU is connected by a suitable serial processor structure to an interactive computer-graphics display system. Typically, the interactive computer-graphics display system comprises a display terminal with resident three-dimensional application software and associated input and output devices, such as X/Y plotters, position control devices (potentiometers, an x-y tablet, or a mouse), and keyboard.

The interactive computer-graphics display system allows the expert operator to view the chemical structures being evaluated in the design process of the

present invention. Graphics and programs are used to select the gaps (Gen. Step 1), and to rank candidates (Gen. Step 3). Essentially, it operates in the same fashion for the single linker embodiment and for the two or more linker embodiments.

For example, during the first general step of the present invention, the computer-graphics interactive display system allows the expert operator to visually display the two naturally aggregated but chemically separate polypeptide chains. Using three dimensional software resident in the computer-graphics display system, the visual representation of the two separate polypeptide chains can be manipulated as desired. For example, the portion of the chain(s) being viewed can be magnified electronically, and such magnification can be performed in a zoom mode. Conversely, the image can be reduced in size, and this reduction can also be done in a reverse zoom mode. The position of the portion of the molecule can be translated, and the displayed molecule can be rotated about any one of the three axes (x, y and z). Specific atoms in the chain can be selected with an electronic pointer. Selected atoms can be labeled with appropriate text. Specific portions of native protein or linker can be identified with color or text or brightness. Unwanted portions of the chain can be erased from the image being displayed so as to provide the expert operator with a visual image that represents only a selected aspect of the chain(s). Atoms selected by pointing or by name can be placed at the center of the three dimensional display; subsequent rotation uses the selected atom as the origin. These and other display aspects provide

the expert operator with the ability to visually represent portions of the chains which increase the ability to perform the structural design process.

One of the modes of the present invention utilizes a serial computational architecture. This architecture using the present equipment requires approximately four to six hours of machine and operator time in order to go through the various operations required for the three general steps for a particular selection of gaps. Obviously, it would be desirable to significantly reduce the time since a considerable portion thereof is the time it takes for the computer system to perform the necessary computational steps.

An alternate embodiment of the present invention utilizes a parallel processing architecture. This parallel processing architecture significantly reduces the time required to perform the necessary computational steps. A hypercube of a large number of nodes can be utilized so that the various linkers that are possible for the selected sites can be rapidly presented to the expert system operator for evaluation.

Since there are between 200 and 300 known protein structures, the parallel processing approach can be utilized. There currently are computers commercially available that have as many as 1,024 computing nodes.

Using a parallel processing approach, the data base of observed peptide structures can be divided into as many parts as there are computing nodes. For example, if there are structures for 195 proteins with 219 amino acids each, one would have structures for 195x218 dipeptides, 195x217 tripeptides, 195x216 tetrapeptides, etc. One can extract all peptides up to

some length n. For example, if n were 30, one would have 195x30x204 peptides. Of course, proteins vary in length, but with 100 to 400 proteins of average length 200 (for example), and for peptide linkers up to length 30 amino acids (or any other reasonable number), one will have between 1,000,000 and 4,000,000 peptide structures. Once the peptides have been extracted and labeled with the protein from which they came, one is free to divide all the peptides as evenly as possible among the available computing nodes.

The parallel processing mode operates as follows. The data base of known peptides is divided among the available nodes. Each gap is sent to all the nodes. Each node takes the gap and tests it against those peptides which have been assigned to it and returns information about any peptides which fit the gap and therefore are candidate linkers. As the testing for matches between peptides and gaps proceeds independently in each node, the searching will go faster by a factor equal to the number of nodes.

A first embodiment of the present invention utilizes a single linker to convert the naturally aggregated but chemically separate heavy and light chains into a single polypeptide chain which will fold into a three dimensional structure very similar to the original structure made of two polypeptide chains.

A second embodiment utilizes two or more linkers to convert the two heavy and light chains into the desired single polypeptide chain. The steps involved in each of these embodiments utilizing the present invention are illustrated in the explanation below.

Once the correct amino acid sequence for a single chain binding protein has been defined by the computer assisted methodology, it is possible, by methods well known to those with skill in the art, to prepare an underlying genetic sequence coding therefor.

In preparing this genetic sequence, it is possible to utilize synthetic DNA by synthesizing the entire sequence de novo. Alternatively, it is possible to obtain cDNA sequences coding for certain preserved portions of the light and heavy chains of the desired antibody, and splice them together by means of the necessary sequence coding for the peptide linker, as described.

Also by methods known in the art, the resulting sequence can be amplified by utilizing well known cloning vectors and well known hosts. Furthermore, the amplified sequence, after checking for correctness, can be linked to promoter and terminator signals, inserted into appropriate expression vectors, and transformed into hosts such as procaryotic or eucaryotic hosts. Bacteria, yeasts (or other fungi) or mammalian cells can be utilized. Upon expression, either by itself or as part of fusion polypeptides, as will otherwise be known to those of skill in the art, the single chain binding protein is allowed to refold in physiological solution, at appropriate conditions of pH, ionic strength, temperature, and redox potential, and purified by standard separation procedures. These would include chromatography in its various different types, known to those with skill in the art.

The thus obtained purified single chain binding protein can be utilized by itself, in detectably la-

belled form, in immobilized form, or conjugated to drugs or other appropriate therapeutic agents, in diagnostic, imaging, biosensors, purifications, and therapeutic uses and compositions. Essentially all uses envisioned for antibodies or for variable region fragments thereof can be considered for the molecules of the present invention.

II. Hardware and Software Environment

A block diagram of the hardware aspects of the present invention is found in Figure 1. A central processing unit (CPU) 102 is connected to a first bus (designated massbus 104) and to a second bus (designated Unibus 106). A suitable form for CPU 102 is a model Vax 11/780 made by Digital Equipment Corporation of Maynard, Massachusetts. Any suitable type of CPU, however, can be used.

Bus 104 connects CPU 102 to a plurality of storage devices. In the best mode, these storage devices include a tape drive unit 106. The tape drive unit 106 can be used, for example, to load into the system the data base of the amino acid sequences whose three dimensional structures are known. A suitable form for tape drive 106 is a Digital Equipment Corporation model TU 78 drive, which operates at 125 inches per second, and has a 1600-6250 bit per inch (BPI) dual capability. Any suitable type of tape drive can be used, however.

Another storage device is a pair of hard disk units labeled generally by reference numeral 108. A suitable form for disk drive 108 comprises two Digital Equipment Corporation RmO5 disk drives having, for

example, 256 Mbytes of storage per disk. Another disk drive system is also provided in the serial processor mode and is labeled by reference numeral 110. This disk drive system is also connected to CPU 102 by bus 104. A suitable form for the disk system 110 comprises three Digital Equipment Corporation model Ra 81 hard disk drives having, for example, 450 Mbytes of storage per disk.

Dynamic random access memory is also provided by a memory stage 112 also connected to CPU 102 by bus 104. Any suitable type of dynamic memory storage device can be used. In the serial processor mode, the memory is made up of a plurality of semi- conductor storage devices found in a DEC model Ecc memory unit. Any suitable type of dynamic memory can be employed.

The disk drives 108 and 110 store several different blocks of information. For example, they store the data base containing the amino acid sequences and structures that are read in by the tape drive 106. They also store the application software package required to search the data base in accordance with the procedures of the present invention. They also store the documentation and executables of the software. The hypothetical molecules that are produced and structurally examined by the present invention are represented in the same format used to represent the protein structures in the data base. Using this format, these hypothetical molecules are also stored by the disk drives 108 and 110 for use during the structural design process and for subsequent use after the process has been completed.

A Digital Equipment Corporation VAX/VMS DEC operating system allows for multiple users and assures file system integrity. It provides virtual memory, which relieves the programer of having to worry about the amount of memory that is used. Initial software was developed under versions 3.0 to 3.2 of the VAX/VMS operating system. The serial processor mode currently is running on version 4.4. DEC editors and FORTRAN compiler were utilized.

The CPU 102 is connected by Bus 106 to a multiplexer 114. The multiplexer allows a plurality of devices to be connected to the CPU 102 via Bus 106. A suitable form for multiplexer 114 is a Digital Equipment Corporation model Dz 16 terminal multiplexer. In the preferred embodiment, two of these multiplexers are used. The multiplexer 114 supports terminals (not shown in Figure 1) and the serial communications (at 19.2 Kbaud, for example) to the computer-graphics display system indicated by the dash lined box 116.

The computer-graphics display system 116 includes an electronics stage 118. The electronic stage 118 is used for receiving the visual image prepared by CPU 102 and for displaying it to the user on a display (typically one involving color) 120. The electronic stage 118 in connection with the associated subsystems of the computer-graphics display system 116 provide for local control of specific functions, as described below. A suitable form of the electronics system 118 is a model PS 320 made by Evans & Sutherland Corp. of Salt Lake, Utah. A suitable form for the display 120 is either a 25 inch color monitor or a 19 inch color monitor from Evans & Sutherland.

Dynamic random access memory 122 is connected to the electronic stage 118. Memory 122 allows the electronic system 118 to provide the local control of the image discussed below. In addition, a keyboard 124 of conventional design is connected to the electronic stage 118, as is an x/y tablet 126 and a plurality of dials 128. The keyboard 124, x/y tablet 126, and dials 128 in the serial processor mode are also obtained from Evans & Sutherland.

The computer generated graphics system 116, as discussed above, receives from CPU 102 the image to be displayed. It provides local control over the displayed image so that specific desired user initiated functions can be performed, such as:

(1) zoom (so as to increase or decrease the size of the image being displayed;

(2) clipping (where the sides, front or back of the image being displayed are removed);

(3) intensity depth queing (where objects further away from the viewer are made dimmer so as to provide a desired depth effect in the image being displayed);

(4) translation of the image in any of the three axes of the coordinate system utilized to plot the molecules being displayed;

(5) rotation in any of the three directions of the image being displayed;

(6) on/off control of the logical segments of the picture. For example, a line connecting the alpha carbons of the native protein might be one logical segment; labels on some or all of the residues of the native protein might be a second logical segment; a trace of the alpha carbons of the linker(s) might be a

third segment; and a stick figure connecting Carbon, Nitrogen, Oxygen, and Sulphur atoms of the linker(s) and adjacent residue of the native protein might be a fourth logical segment. The user seldom wants to see all of these at once; rather the operator first becomes oriented by viewing the first two segments at low magnification. Then the labels are switched off and the linker carbon trace is turned on. Once the general features of the linker are seen, the operator zooms to higher magnification and turns on the segments which hold more detail;

(7) selection of atoms in the most detailed logical segment. Despite the power of modern graphics, the operator can be overwhelmed by too much detail at once. Thus the operator will pick one atom and ask to see all amino acids within some radius of that atom, typically 6 Angstroms, but other radii can be used. The user may also specify that certain amino acids will be included in addition to those that fall within the specified radius of the selected atom;

(8) changing of the colors of various portions of the image being displayed so as to indicate to the viewer particular information using visual queing.

As stated above, the serial processor mode of the present invention currently is running the application software on version 4.4 of the Vax/Vms operating system used in conjunction with CPU 102. The application programs were programmed using the FLECS (FORTRAN Language with Extended Control Sections) programming language written in 1974 by Terry Beyer of the University of Oregon, Eugene, Oregon. FLECS is a FORTRAN preprocessor, which allows more logical programming.

All of the code used in the serial processor mode was developed in FLECS. It can be appreciated, however, that the present invention encompasses other operating systems and programming languages.

The macromolecules displayed on color display 120 of the computer-graphics display system 116 utilize an extensively modified version of version 5.6 of FRODO. FRODO is a program for displaying and manipulating macromolecules. FRODO was written by T.A. Jones at Max Planck Institute for Biochemistry, Munich, West Germany, for building or modeling in protein crystallography. FRODO version 5.6 was modified so as to be driven by command files; programs were then written to create the command files. It is utilized by the electronic stage 118 to display and manipulate images on the color display 120. Again, any suitable type of program can be used for displaying and manipulating the macromolecules, the coordinates of which are provided to the computer-graphics display system 116 by the CPU 102.

Design documentation and memos were written using PDL (Program Design Language) from Caine, Farber & Gordon of Pasadena, California. Again, any suitable type of program can be used for the design documents and memos.

Figure 2 shows a block diagram for an improved version of the hardware system of the present invention. Like numbers refer to like items of Figure 1. Only the differences between the serial processor mode system of Figure 1 and the improved system of Figure 2 are discussed below.

The CPU 102' is the latest version of the Vax 11/780 from Digital Equipment Corporation. The latest processor from DEC in the VAX product family is approximately ten times faster than the version shown in the serial processor mode of Figure 1.

Instead of the two Rm05 disk drives 108 of Figure 1, the embodiment of Figure 2 utilizes five RA 81 disk drive units 110'. This is to upgrade the present system to more state of the art disk drive units, which provide greater storage capability and faster access.

Serial processor 106 is connected directly to the electronic stage 118' of the computer-graphics display system 116. The parallel interface in the embodiment of Figure 2 replaces the serial interface approach of the serial processor mode of Figure 1. This allows for faster interaction between CPU 102' and electronic stage 118' so as to provide faster data display to the expert operator.

Disposed in front of color display 120 is a stereo viewer 202. A suitable form for stereo viewer 202 is made by Terabit, Salt Lake City, Utah. Stereo viewer 202 would provide better 3-D perception to the expert operator than can be obtained presently through rotation of the molecule.

In addition, this embodiment replaces the FRODO macromolecule display programs with a program designed to show a series of related hypothetical molecules. This newer program performs the operations more quickly so that the related hypothetical molecules can be presented to the expert operator in a short enough time that makes examination less burdensome on the operator.

The programs can be modified so as to cause the present invention to eliminate candidates in the second general step where obvious rules have been violated by the structures that are produced. For example, one rule could be that if an atom in a linker comes closer than one Angstrom to an atom in the native structure the candidate would be automatically eliminated.

In addition, the surface accessibility of molecules could be determined and a score based on the hydrophobic residues in contact with the solvent could be determined. After the hydrophobic residues have been calculated, the candidates could be ranked so that undesired candidates could automatically be eliminated. The protein is modeled in the present invention without any surrounding matter. Proteins almost always exist in aqueous solution; indeed, protein crystals contain between 20% and 90% water and dissolved salts which fill the space between the protein molecules. Certain kinds of amino acids have sidechains which make favorable interactions with aqueous solutions (serine, threonine, arginine, lysine, histidine, aspartic acid, glutamic acid, proline, asparagine, and glutamine) and are termed hydrophilic. Other amino acids have side chains which are apolar and make unfavorable interactions with water (phenylalanine, tryptophan, leucine, isoleucine, valine, methionine, and tyrosine) and are termed hydrophobic. In natural proteins, hydrophilic amino acids are almost always found on the surface, in contact with solvent; hydrophobic amino acids are almost always inside the protein in contact with other hydrophobic amino acids.

The remaining amino acids (alanine, glycine, and cysteine) are found both inside proteins and on their surfaces. The designs of the present invention should resemble natural proteins as much as possible, so hydrophobic residues are placed inside and hydrophilic residues are placed outside as much as possible.

Programs could be utilized to calculate an energy for each hypothetical structure. In addition, programs could make local adjustments to the hypothetical molecules to minimize the energy. Finally, molecular dynamics could be used to identify particularly unstable parts of the hypothetical molecule. Although existing programs could calculate a nominal energy for each hypothetical structure, it has not yet been demonstrated that such calculations can differentiate between sequences which will fold and those that will not. Energy minimization could also be accomplished with extant programs, but energy minimization also can not differentiate between sequences which will fold and those that will not. Molecular dynamics simulations currently cannot be continued long enough to simulate the actual folding or unfolding of a protein and so cannot distinguish between stable and unstable molecules.

Two megabytes of storage 128' in the computer generated display system 116 is added so that several different molecules can be stored at the display level. These molecules then can be switched back and forth on the color display 120 so that the expert operator can sequentially view them while making expert decisions. The parallel interface that is shown in Figure 2 would allow the coordinates to be trans-

ferred faster from the CPU 102' to the electronics stage 118' of the computer generated display system 116.

The parallel processing architecture embodiment of the present invention is described below in Section V. This parallel architecture embodiment provides even faster analysis and display.

III. Single Linker Embodiment

This first embodiment of the present invention determines and displays possible chemical structures for using a single linker to convert the naturally aggregated but chemically separate heavy and light polypeptide chains into a single polypeptide chain which will fold into a three dimensional structure very similar to the original structure made of two polypeptide chains.

A. Plausible Site Selection

There are two main goals of the plausible site selection step 302 of the present invention shown in very generalized block diagram form in Figure 3. The first goal is to select a first plausible site on the first chain that is the minimum distance from the second plausible site on the second chain. The first point on the first chain and the second point on the second chain comprise the plausible site.

The second goal of the site selection is to select plausible sites that will result in the least loss of native protein. Native protein is the original protein composed of the two aggregated polypeptide chains of the variable region. It is not chemically possible to convert two chains to one without altering some of

the amino acids. Even if only one amino acid was added between the carboxy terminal of the first domain and the amino terminal of the second domain, the charges normally present at these termini would be lost. In the variable regions of antibodies, the terminii of the H and L chains are not very close together. Hypothetical linkers which join the carboxy terminus of one chain to the amino terminus of the other do not resemble the natural variable region structures. Although such structures are not impossible, it is more reasonable to cut away small parts of the native protein so that compact linkers which resemble the native protein will span the gap. Many natural proteins are known to retain their structure when one or more residues are removed from either end.

In the present embodiment, only a single linker (amino acid sequence or bridge for bridging or linking the two plausible sites to form a single polypeptide chain) is used. Figure 4 shows in block diagram form the steps used to select plausible sites in the single linker. The steps of Figure 4 are a preferred embodiment of step 302 of Figure 3.

A domain 1 is picked in a step 402 (see Figure 4). A schematic diagram of two naturally aggregated but chemically separate polypeptide chains is shown in Figure 5A. For purposes of illustration, assume that L is the light chain of the antibody variable region (the first polypeptide chain) and is domain 1. As shown in Figure 5A, light chain L is on the left side, and heavy chain H is on the right side.

The next step 404 is to pick the domain 2, which, as indicated, is the heavy chain H of the antibody variable region on the right side of Figure 5A.

The linker that will be selected will go from domain 1 (the light chain L) towards domain 2 (heavy chain, H). As the linker will become part of the single polypeptide chain, it must have the same directionality as the polypeptides it is linking; i.e. the amino end of the linker must join the carboxy terminal of some amino acid in domain 1, and the carboxy terminal of the linker must join the amino terminal of some residue in domain 2. A starting point (first site) on domain 1 is selected, as represented by step in 406 in Figure 4. The starting point is chosen to be close to the C (C for carboxy) terminal of domain 1, call this amino acid tau 1. It is important to pick tau 1 close to the C terminal to minimize loss of native protein structure. Residue tau 1 is shown schematically in two dimensions in figure 6A; it is also shown in figure 6B where it is presented in a two-dimensional representation of the naturally aggregated but chemically separate H and L polypeptide chains.

Next, the final point (second site) close the N (N for amino) terminal of domain 2 is selected, as indicated by step 408 of Figure 4. The final site is an amino acid of domain 2 which will be called sigma 1. It is important that amino acid sigma 1 be close to the N terminal of domain 2 to minimize loss of native protein structure. Amino acid sigma 1 is shown schematically in figure 6A and in the more realistic representation of figure 6B.

Figure 7 shows in simplified form the concept that the linker goes from a first site at amino acid tau 1 in domain 1 to a second site at amino acid sigma 1 in

domain 2. There are a plurality of possible first sites and a plurality of second sites, as is shown in figure 7. A computer program prepares a table which contains for each amino acid in domain 1 the identity of the closest amino acid in domain 2 and the distance. This program uses the position of the alpha carbon as the position of the entire amino acid. The expert operator prepares a list of plausible amino acids in domain 1 to be the first site, tau 1, and a list of plausible amino acids in domain 2 to be the second site, sigma 1. Linkers are sought from all plausible sites tau 1 to all plausible sites sigma 1. The expert operator must exercise reasonable judgement in selecting the sites tau 1 and sigma 1 in deciding that certain amino acids are more important to the stability of the native protein than are other amino acids. Thus the operator may select sites which are not actually the closest.

The complete designed protein molecule in accordance with the present invention consists of the domain 1 (of the light chain L) up to the amino acid tau 1, the linker, as shown by the directional-line in Figure 8A and in Figure 8B, and the domain 2 from amino acid sigma 1 to the C terminus of the heavy chain, H. As shown in Figures 8A and 8B, in the representative example, this results in the following loss of native protein.

The first loss in native protein is from the residue after residue tau 1 to the C terminus of domain 1 (light chain L). The second loss of native protein is from the N terminus of domain 2 (heavy chain, H) to the amino acid before sigma 1.

As is best understood from Figure 8A, the introduction of linker 1 produces a single polypeptide chain from the two naturally aggregated chains. The polypeptide chain begins with the N terminal of domain 1. Referring now to Figure 8B, the chain proceeds through almost the entire course of the native light chain, L, until it reaches amino acid tau 1. The linker then connects the carboxy terminal of a very slightly truncated domain 1 to residue sigma 1 in the very slightly truncated domain 2. Since a minimum amount of native protein is eliminated, and the linker is selected to fit structurally as well as possible (as described below in connection with general steps 2 and 3 of the present invention), the resulting single polypeptide chain has a very high probability (several orders of magnitude greater than if the linker was selected randomly) to fold into a three-dimensional structure very similar to the original structure made of two polypeptide chains.

The single polypeptide chain results in a much more stable protein which contains a binding site very similar to the binding site of the original antibody. In this way a single polypeptide chain can be engineered from the naturally occuring two-polypeptide chain variable region, so as to create a polypeptide of only one chain, but maintaining the binding site of the antibody.

In the current mode of the present invention, the expert operator selects the sites with minimal help from the computer. The computer prepares the table of closest-residue-in-other-domain. The computer can provide more help in the following ways.

(1) Prepare a list of conserved and variable residues for variable regions of antibodies (Fv region). Residues which vary from Fv to Fv would be much better starting or ending sites for linkage than are residues which are conserved over many different Fv sequences.

(2) Prepare a list of solvent accessibilities. Amino acids exposed to solvent can be substituted with less likelihood of destabilizing the native structure than amino acids buried within the native structure. Exposed amino acids are better choices to start or end linkage.

With respect to each of the plurality of possible first sites (on domain 1 or light chain L) there are available a plurality of second sites (on domain 2 or heavy chain H) (See Figures 7 and 8A). As the second site is selected closer to the N terminus of domain 2, the distance to any of the plausible first sites increases. Also, as the first site is selected closer to the C terminus of domain 1 the distance to any of the plausible second sites increases. It is this tension between shortness of linker and retention of native protein which the expert operator resolves in choosing gaps to be linked. The penalty for including extra sites in the list of gaps are:

(1) searching in general step 2 will be slower; and

(2) more candidates will pass from step 2 many of which must be rejected in step 3. As step 3 is currently a manual step, this is the more serious penalty.

Figure 8B shows diagramatically by a directional arrow the possible links that can occur between the various

sites near the C terminal of domain 1 and the various sites near the N terminal of domain 2.

B. Selection of Candidates

In the second of the three general steps of the present invention as used in the single linker embodiment, plausible candidates for linking the site 1 on domain 1 with site 2 on domain 2 are selected from a much larger group of candidates. This process of winnowing out candidates results in the expert operator and/or expert system having a relatively small group of candidates to rank from most plausible to least plausible in the third general step of the present invention, as described in subsection C below.

Currently, there are approximately 250 protein structures, determined at 2.0 A or higher resolution, in the public domain. The structures of these very complicated molecules are determined using sophisticated scientific techniques such as X-ray crystallography, neutron diffraction, and nuclear magnetic resonance. Structure determination produces a file of data for each protein. The Brookhaven Protein Data Bank (BPDB) exemplifies a repository of protein structural information. Each file in BPDB contains many records of different types. These records carry the following information:

(1) Name of the protein and standard classification number,

(2) Organism from which protein was obtained,

(3) Name and address of contributor,

(4) Amino-acid sequence of each polypeptide chain, if known,

(5) Connectivity of disulfides, if any,

(6) Names and connectivities of any prosthetic groups, if any,

(7) References to literature,

(8) Transformation from reported coordinates to crystallographic coordinates,

(9) Coordinates of each atom determined.

There is at least one record for each atom for which a coordinate was determined. Some parts of some proteins are disordered and do not diffract X-rays, so no sensible coordinates can be given. Thus there may be amino acids in the sequence for which only some or none of the atoms have coordinates. Coordinates are given in Angstrom units (100,000,000 A = 1 cm) on a rectangular Cartesian grid. As some parts of a protein may adopt more than one spatial configuration, there may be two or more coordinates for some atoms. In such cases, fractional occupancies are given for each alternative position. Atoms move about, some more freely than others. X-ray data can give an estimate of atomic motion which is reported as a temperature (a.k.a. Debye-Waller) factor.

Any other data base which included, implicitly or explicitly, the following data would be equally useful:

(1) Amino acid sequence of each polypeptide chain.

(2) Connectivity of disulfides, if any,

(3) Names and connectivities of any prosthetic groups, if any,

(4) Coordinates (x, y, z) of each atom in each observed configuration.

(5) Fractional occupancy of each atom,

(6) Temperature factor of each atom.

Proteins usually exist in aqueous solution. Although protein coordinates are almost always determined for proteins in crystals, direct contacts between proteins are quite rare. protein crystals contain from 20% to 90% water by volume. Thus one usually assumes that the structure of the protein in solution will be the same as that in the crystal. It is now generally accepted that the solution structure of a protein will differ from the crystal structure only in minor details. Thus, given the coordinates of the atoms, one can calculate quite easily the solvent accessibility of each atom.

In addition, the coordinates implicitly give the charge distribution throughout the protein. This is of use in estimating whether a hypothetical molecule (made of native protein and one or more linkers) will fold as designed. The typical protein whose structure is known comprises a chain of amino acids (there are 21 types of amino acids) in the range of 100 to 300 amino acids.

Each of these amino acids alone or in combination with the other amino acids as found in the known protein molecule can be used as a fragment to bridge the two sites. The reason that known protein molecules are used is to be able to use known protein fragments for the linker or bridge.

Even with only 250 proteins of known structure, the number of possible known fragments is very large. A linker can be from one to twenty or thirty amino acids long. Let "Lmax" be the maximum number of amino acids allowed in a linker, for example, Lmax might be

25. Consider a protein of "Naa" amino acids. Proteins have Naa in the range 100 to 800, 250 is typical. From this protein one can select Naa-1 distinct two-amino-acid linkers, Naa-2 distinct three-amino-acid linkers,...and (Naa+1-Lmax) distinct linkers containing exactly Lmax amino acids. The total number of linkers containing Lmax or fewer linkers is "Nlink,"

$$Nlink = \sum_{j=1,Lmax} (Naa+1-j)$$

$$= Naa \times (Lmax) - (Lmax \times Lmax)/2 + Lmax/2$$

If Naa is 250 and Lmax is 25, Nlink will be 5975. If the number of known proteins is "Nprot," then the total number of linkers, "Nlink_total" will be

$$Nlink\_total = \sum_{k=1,Nprot} \sum_{j=1,Lmax} (Naa(k)+1-j)$$

$$= \sum_{k=1,Nprot} [Naa(k)x(Lmax) - (LmaxxLmax)/2+Lmax/2]$$

$$= Nprotx(Lmax/2-Lmax \times Lmax)/2 + Lmax \times \sum_{K=1,Nprot} Naa(k)$$

Where Naa(k) is the number of amino acids in the kth protein. With 250 proteins, each containing 250 amino acids (on average), and Lmax set to 25, Nlink_total is 1,425,000.

This is the number of linkers of known structure. If one considers the number of possible amino acid sequences up to length Lmax (call it "Nlink_possible"), it is much larger.

$$\text{Nlink\_possible} = \sum_{J = 1,\text{Lmax}} 20^J$$

For Lmax = 25

Nlink_possible = 353, 204, 547, 368, 421, 052, 631, 578, 947, 368, 420

$$= 3.53 * 10^{32}$$

Using known peptide fragments thus reduces the possibilities by twenty-six orders of magnitude. Appropriate searching through the known peptide fragments reduces the possibilities a further five orders of magnitude.

Essentially, the present invention utilizes a selection strategy for reducing a list of possible candidates. This is done as explained below in a preferred form in a three step process. This three step process, as is illustrated in the explanation of the each of the three steps of the process, significantly reduces the computer time required to extract the most promising candidates from the data base of possible candidates. This should be contrasted with a serial search throughout the entire data base of candidates,

which would require all candidates to be examined in total. The present invention examines certain specific parameters of each candidate, and uses these parameters to produce subgroups of candidates that are then examined by using other parameters. In this way, the computer processing speed is significantly increased.

The best mode of the present invention uses a protein data base created and supplemented by the Brookhaven National Laboratory in Upton, Long Island, New York. This data base is called the Brookhaven Protein Data Base (BPDB). It provides the needed physical and chemical parameters that are needed by the present invention. It should be understood, that the candidate linkers can be taken from the Brookhaven Protein Data Base or any other source of three-dimensional protein structures. These sources must accurately represent the proteins. In the current embodiment, X-ray structures determined at resolution of 2.5A or higher and appropriately refined were used. Each peptide is replaced (by least-squares fit) by a standard planar peptide with standard bond lengths and angles. Peptides which do not accurately match a standard peptide (e.g. cis peptides) are not used to begin or end linkers, but may appear in the middle.

Each sequence up to some maximum number of amino acids (Lmax) is taken as a candidate. In the preferred embodiment, the maximum number of amino acids (Lmax) is set to 30. However, the present invention is not limited to this number, but can use any maximum number that is desired under the protein engineering circumstances involved.

1. **Selecting Candidates with Proper Distance Between the N Terminal and the C Terminal.**

The first step in the selection of candidates step is to select the candidate linkers with a proper distance between the N terminal and the C terminal from all of the candidate linkers that exist in the protein data base that is being used. Figure 9 shows in block diagram form the steps that make up this candidate selection process utilizing distance as the selection parameter.

Referring to Figure 9, a standard point relative to the peptide unit at the first site is selected, as shown by block 902.

A standard point relative to the peptide unit in the second site is also picked, as indicated by a block 904. Note that in the best mode the geometric centers of the peptide units of the first and second sites are used, but any other standard point can be utilized, if desired.

The distance between the standard points of the two peptides at the first and second sites defining the gap to be bridged by the linker is then calculated, as indicated by block 906. This scalar distance value is called the Span of the gap. Note that this scalar value does not include any directional information.

Next, as indicated by a step 908, the distance between the ends of the possible linker candidates are calculated. The distance between the ends of a particular candidate is called the span of the candidate. Note that each possible linker candidate has a span of the candidate scalar value.

The final step in the distance selection candidate selection process is that of a step 910. In step 910, candidates are discarded whose span of the candidate values differ from the span of the gap value by more than a preselected amount (this preselected amount is Max LSQFIT error). In the best mode of the present invention, the preselected amount for Max LSQFIT error is 0.50 Angstroms. However, any other suitable value can be used.

The preceding discussion has been for a single gap. In fact, the expert user often selects several gaps and the search uses all of them. The span of each candidate is compared to the span of each gap until it matches one, within the preset tolerance, or the list of gaps is exhausted. If the candidate matches none of the gaps, it is discarded. If it matches any gap it is carried to the next stage.

The inventors have determined that the use of the distance as the first parameter for discarding possible linker candidates results in a significant reduction in the number of possible candidates with a minimum amount of computer time that is needed. In terms of the amount of reduction, a representative example (using linkers up to 20 amino acids) starts out with 761,905 possible candidates that are in the protein data base. This selection of candidates using the proper distance parameter winnows this number down to approximately 63,727 possible candidates. As is discussed below, the distance selection operation requires much less computer time than is required by the other two steps which make up this selection step 304.

The result of this selection of candidates according to proper distance is a group (called a first group of candidates) which exhibit a proper length as compared to the gap that is to be bridged or linked. This first group of candidates is derived from the protein data base using the distance criteria only.

2. Selecting Candidates with Proper Direction from N Terminal to C Terminal

This substep essentially creates a second group of possible candidates from the first group of possible candidates which was produced by the distance selection substep discussed in connection with Figure 9. The second group of candidates is selected in accordance with the orientation of the C terminal residue (i.e. the final residue) of the linker with respect to the N terminal residue (i.e. the initial residue) which is compared to the orientation of the C terminal residue (i.e. the second site) of the gap with respect to the N terminal residue (i.e. the first site). See Figure 20B. In this way, this direction evaluation determines if the chain of the linker ends near the second site of the gap, when the amino terminal amino acid of the linker is superimposed on the first site of the gap so as to produce the minimum amount of unwanted molecular distortion.

Referring now to Figure 10, the first step used in producing the second group of possible candidates is a step 1002. In step 1002 a local coordinate system is established on the N terminal residue of one of the selected gaps. For example, one might take the local X-axis as running from the first alpha carbon of the N

terminal residue to the second alpha carbon of the N terminal residue, with the first alpha carbon at the origin - the second alpha carbon on the plus X-axis. The local Y-axis is selected so that the carbonyl oxygen lies in the xy plane with a positive y coordinate. The local Z-axis is generated by crossing X into Y. Next, as indicated by step 1004, a standard reference point in the C terminal residue of the gap is located and its spherical polar coordinates are calculated in the local system. The standard reference point could be any of the atoms in the C terminal peptide (throughout this application, peptide, residue, and amino acid are used interchangeably) or an average of their positions. Steps 1002 and 1004 are repeated for all gaps in the list of gaps. As indicated by step 1006, a local coordinate system is established on the N terminal residue of one of the candidates. This local coordinate system must be established in the same manner used for the local coordinate systems established on each of the gaps. Various local systems could be used, but one must use the same definition throughout. In step 1008, the standard reference point is found in the C terminal residue of the current candidate. This standard point must be chosen in the same manner used for the gaps. The spherical polar coordinates of the standard point are calculated in the local system of the candidate. (This use of local coordinate system is completely equivalent to rotating and translating all gaps and all candidates so that their initial peptide lies in a standard position at the origin.) In step 1010, the spherical polar coordinates of the gap vector (r, theta, phi) are compared

to the spherical polar coordinates of the candidate vector (r, theta, phi). In step 1012 a preset threshhold is applied, if the two vectors agree closely enough, then one proceeds to step 1014 and enrolls the candidate in the second group of candidates. Currently, this preset threshhold is set to 0.5 A, but other values could be used. From step 1014, one skips forward to step 1022, vide infra. On the other hand, if the vectors compared in step 1012 are not close enough, one moves to the next gap vector in the list, in step 1016. If there are no more gaps, one goes to step 1018 where the candidate is rejected. If there are more gaps, step 1020 increments the gap counter and one returns to step 1010. From steps 1014 or 1018 one comes to step 1022 where one tests to see if all candidates have been examined. If not, step 1024 increments the candidate counter and one returns to step 1006. If all candidates have been examined, one has finished, step 1026.

Figure 11 shows the concept of comparing the direction of the gap to the direction of the candidate.

The inventors have determined that in the example discussed above where 761,905 possible candidates are in the protein data base, the winnowing process in this step reduces the approximate 63,727 candidates in the first group to approximately 50 candidates in the second group. The inventors have also determined that as referenced to the units of computer time referred to above in connection with the scalar distance parameter, it takes approximately 4 to 5 computer units of time to perform the selection of this step. Thus, it can be appreciated that it preserves computer time to

perform the distance selection first, and the direction selection second since the direction selection process takes more time than the distance selection process.

3. Selecting Candidates with Proper Orientation at Both Termini

In this step, the candidates in the second group of step 1016 of Figure 10 are winnowed down to produce a third group of plausible candidates using an evaluation of the relative orientation between the peptide groups at either end of the candidate, compared to the relative orientation between the peptide groups at either end of the gap. In a step 1201, (Figure 12) decide that a peptide will be represented by 3, 4, or 5 atoms (vide infra). Specifically, in a step 1202, one of the candidates in the second group (step 1014) is selected for testing. In a step 1204, three to five atoms in the first peptide are selected to define the orientation of the first peptide. So long as the atoms are not collinear, three atoms is enough, but using four or five atoms makes the least-squares procedure which follows over-determined and therefore compensates for errors in the coordinates. For example, assume selection of four atoms: C alpha, C, N, and C beta. Next, in a step 1206, one selects the corresponding 3,4, or 5 atoms from the final peptide of the selected candidate. These 6, 8, or 10 atoms define a three-dimensional object. In a step 1208, select one of the gaps. Select the corresponding 6, 8, or 10 atoms from the gap. In a step 1210, least-squares fit the atoms from the candidate to the atoms from the gap. This least-squares fit allows degrees

of freedom to superimpose the two three-dimensional objects. Assume that one object is fixed and the other is free to move. Three degrees of freedom control the movement of the center of the free object. Three other degrees of freedom control the orientation of the free object. In a step 1212, the result of the least-square fit is examined. If the Root-Mean-Square (RMS) error is less than some preset threshhold, the the candidate is a good fit for the gap being considered and is enrolled in the third group in a step 1214. If, on the other hand, the RMS error is greater than the preset threshhold, one checks to see if there is another gap in the list in a step 1216. If there is, one selects the next gap and returns to step 1208. If there are no more gaps in the list, then the current candidate from the second group is rejected in step 1218. In step 1220, one checks to see if there are more candidates in the second group; if so, a new candidate is selected and one returns to step 1201. If there are no more candidates, one is finished (step 1222). Again referring to a representative case, where linkers of length up to twenty amino acids were sought for a single gap with separation 12.7 A, the protein data bank contained 761,905 potential linkers. Of these, 63,727 passed the distance test. The direction test removed all but 50 candidates. The orientation test passed only 1 candidate with RMS error less than or equal to 0.5 A. There were two additional candidates with RMS error between 0.5 A and 0.6 A. Moreover, the inventors have determined that it takes about 25 units of computer time to evaluate each candidate in group 2 to decide whether they should be

selected for group 3. It can be appreciated now that the order selected by the inventors for the three steps of winnowing the candidates has been selected so that the early steps take less time per candidate than the following steps. The order of the steps used to select the candidate can be changed, however, and still produce the desired winnowing process. Logically, one might even omit steps one and two and pass all candidates through the least-squares process depicted in Figure 12 and achieve the same list of candidates, but at greater cost in computing. This may be done in the case of parallel processing where computer time is plentiful, but memory is in short supply.

Another approach (not illustrated) for determining whether the proper orientation exists between the ends of the candidate, is to examine only the atoms at the C terminal of the candidate as compared to the atoms at the final peptide of the gap. In step 2, the inventors aligned the first peptide of the candidate with the first peptide in the gap. Having done this, one could merely compare the atoms at the C terminal of the candidate with the atoms of the second peptide of the gap. This approach is inferior to that discussed above because all the error appears at the C terminus, while the least-squares method discussed above distributes the errors evenly.

C. Ranking and Eliminating Candidates.

As shown in Figure 3, the third general step in the present invention is that of ranking the plausible candidates from most plausible to least plausible, and eliminating those candidates that do not appear to be plausible based on criteria utilized by an expert operator and/or expert system.

In the best mode, the candidates in the third group (step 1214) are provided to the expert operator, who can sequentially display them in three dimensions utilizing the computer-graphics display system 116. The expert operator then can make decisions about the candidates based on knowledge concerning protein chemistry and the physical relationship of the plausible candidate with respect to the gap being bridged. This analysis can be used to rank the plausible candidates in the third group from most plausible to least plausible. Based on these rankings, the most plausible candidates can be selected for genetic engineering.

As noted above in connection with the illustrative example, there are typically few (under 100) candidates which make it to the third group of step 1214. Consequently, a moderately expert operator (one having a Bachelor of Science degree in chemistry, for example), can typically winnow down this number of plausible candidates to a group of 10 to 15. Thereafter, a more expert operator and/or expert system can further winnow down the number. In this way, only a very few of the plausible candidates needs to be tested in practice as compared to the hundreds, thousands or more of candidates that would have to be tested if no selection process like that of the present invention was used. This speeds up the process of engineering the single chain molecules by orders of magnitude, while reducing costs and other detriments by orders of magnitude as well.

In certain situations, however, automatic ranking in this third general step may be warranted. This could occur, for example, where the expert operator

was presented with quite a few candidates in the third group, or where it is desired to assist the expert operator in making the ranking selections and eliminating candidates based on prior experience that has been derived from previous engineering activities and/or actual genetic engineering experiments.

Referring now to Figure 13, a coordinate listing of the hypothetical molecule (candidate) is automatically constructed, as is indicated by a block 1302. The expert operator can then display using a first color the residues from domain 1 of the native protein. Color display 120 can provide a visual indication to the expert operator of where the residues lie in domain 1. This is indicated by a block 1304.

The expert operator then can display on color display 120 the residues from domain 2 of the native protein using a second color, as is indicated by a block 1306. The use of a second color provides a visual indication to the user which assists in distinguishing the residues from domain 1 from the residues from domain 2.

The linker (candidate) being ranked can be displayed in a selected color, which color can be different from the first color of step 1304 and/or the second color from step 1306. Again, by using this visual color indication, the expert operator can distinguish the residues of domain 1 and 2 of the native protein. This display of the linker candidate is indicated by a block 1308.

The initial picture on the color display 120 provided to the expert operator typically shows the alpha carbons for all of the residues. This is indicated by

a block 1310. In addition, the initial picture shows the main-chain and side-chains for residues and linkers and one residue before the linker and one residue after the linker. This is indicated by a block 1312.

The expert operator can also cause any of the other atoms in the native protein or linker candidate to be drawn at will. The molecule can be rotated, translated, and enlarged or reduced, by operator command, as was discussed generally in connection with the computer-graphics display system 116 above. The block diagram of Figure 13 indicates that each of the steps just discussed are accomplished in serial fashion. However, this is only for purposes of illustration. It should be understood that the operator can accomplish any one or more of these steps as well as other steps at will and in any sequence that is desired in connection with the ranking of the plausible candidates in group 3.

The expert operator and/or expert system utilized in this third general step in ranking the candidates from most plausible to least plausible and in eliminating the remaining candidates from group 3, can use a number of different rules or guidelines in this selection process. Representive of these rules and guidelines are the following which are discussed in connection with Figure 14. Note that the blocks in Figure 14 show the various rules and/or criteria, which are not necessarily utilized in the order in which the boxes appear. The order shown is only for purposes of illustration. Other rules and/or criteria can be utilized in the ranking process, as well.

As shown in step 1402, a candidate can be rejected if any atom of the linker comes closer than a minimum allowed separation to any retained atom of the native protein structure. In the best mode, the minimum allowed separation is set at 2.0 Angstroms. Note that any other value can be selected. This step can be automated, if desired, so that the expert operator does not have to manually perform this elimination process.

A candidate can be penalized if the hydrophobic residues have high exposure to solvent, as is indicated by a block 1404. The side chains of phenylananine, tryptophan, tyrosine, leucine, isoleucine, methionine, and valine do not interact favorably with water and are called hydrophobic. Proteins normally exist in saline aqueous solution; the solvent consists of polar molecules (H_2O) and ions.

A candidate can be penalized when the hydrophilic residues have low exposure to solvent. The side chains of serine, threonine, aspartic acid, glutamic acid, asparagine, glutamine, lysine, arginine, and proline do interact favorably with water and are called hydrophilic. This penalization step for hydrophilic residues is indicated by a block 1406.

A candidate can be promoted when hydrophobic residues have low exposure to solvent, as is indicated by a block 1408.

A candidate can be promoted when hydrophilic residues have high exposure to solvent, as indicated by a block 1410.

A candidate can be penalized when the main chain fails to form hydrogen bonds, as is indicated by a block 1412.

A candidate can be penalized when the main chain makes useless excursions into the solvent region. Useless excursions are those which do not make any evident interaction with the retained native protein. This is indicated by a block 1414.

A candidate can be promoted when the main chain forms a helix, as is indicated by a block 1416. Hilices are self-stabilizing. Thus a linker which is helical will be more stable because its main-chain polar atoms (O and N) will form hydrogen bonds within the linker.

As is indicated by a block 1418, a candidate can be promoted when the main chain forms a beta sheet which fits against existing beta sheets. The strands of beta sheets stabilize each other. If a linker were found which was in a beta-sheet conformation such that it would extend an existing beta sheet, this interaction would stabilize both the linker and the native protein.

Another expert design rule penalizes candidates which have sterically bulky side chains at undesirable positions along the main chain. Furthermore, it is possible to "save" a candidate with a bulky side chain by replacing the bulky side chain by a less bulky one. For example if a side chain carries a bulky substituent such as leucine or isoleucine, a possible design step replaces this amino acid by a glycine, which is the least bulky side chain.

Other rules and/or criteria can be utilized in the selection process of the third general step 306, and the present invention is not limited to the rules and/or criteria discussed. For example, once the

linker has been selected it is also possible to add, delete, or as stated, modify one or more amino acids therein, in order to accomplish an even better 3-D fit.

IV. Double and Multiple Linker Embodiments

Section III above described the single linker embodiment in accordance with the present invention. This section describes double linker and multiple linker embodiments in accordance with the present invention. For brevity purposes, only the significant differences between this embodiment and the single linker embodiment will be described here and/or illustrated in separate figures. Reference should therefore be made to the text and figures that are associated with the single linker embodiment

A. Plausible Site Selection.

The two main goals of minimizing distance between the sites to be linked and the least loss of native protein apply in the site selection in the double and multiple linker embodiments as they did apply in the single linker embodiment discussed above.

Figure 15A shows a simplified two dimensional representation of the use of two linkers to create the single polypeptide chain from the two naturally aggregated but chemically separate polypeptide chains. Figure 15B shows in two dimensions a three dimensional representation of the two chains of Figure 15A. Referring now to Figures 15A and B, the first step in de-

termining suitable sites is to find a site in domain 1 which is close to either the C or N terminus of domain 2. For purposes of illustration, and as is shown in Figures 15A and 15B, it is assumed that the most promising location is the C terminus of domain 2. The residue in domain 1 is called Tau 1, while the residue in domain 2 is called Sigma 1.

Figures 16A and 16B are respectively two dimensional simplified plots of the two chains, and two dimensional plots of the three dimensional representation of the two chains. They are used in connection with the explanation of how plausible sites are selected for the second linker in the example situation.

The first step in connection with finding plausible sites for the second linker is to find a residue in domain 1 that is before Tau 1 in the light chain. This residue is called residue Tau 2. It is shown in the top portion in Figure 16A, and in the right middle portion in Figure 16B.

The next step in the site selection process for the second linker is to find a residue in domain 2 near the N terminus of domain 2. This residue is called residue Sigma 2. Reference again is made to Figures 16A and B to show the location of Sigma 2.

The second linker (linker 2) thus runs from Tau 2 to Sigma 2. This is shown in Figures 17A and 17B. Note that the chain that is formed by these two linkers has the proper direction throughout.

Figure 18 shows in two dimensional simplified form the single polypeptide chain that has been formed by the linking of the two independent chains using the two linkers. Note that the approach outlined above

resulted in the minimal loss of native protein. The completely designed protein is shown in Figure 17 and consists of domain 1 from the N terminal to Tau 2, linker 2, domain 2 from Sigma 2 to Sigma 1, linker 1, and domain 1 from Taul to the C terminus. The arrows that are shown in Figure 17 indicate the direction of the chain.

Figure 17 shows that the residues lost by the utilization of the two linkers are: (a) from the N terminus of domain 2 up to the residue before Sigma 2; and (b) from the residue after Sigma 1 to the C terminus of domain 2; and (c) from the residue after Tau 2 to the residue before Tau 1 of domain 1.

If one of the linkers in the two linker case is very long, one could link from Tau 2 to a residue in domain 2 after Sigma 1. A third linker (not shown) would then be sought from a residue near the C terminal of domain 2 to a residue near the N terminal of domain 2.

Additionally, one could use two linkers to reconnect one of the domains in such a way that a single linker or a pair of linkers would weld the two domains into one chain.

B. Candidate Selection and Candidate Rejection Steps

Ranking of linkers in the multilinker cases follows the same steps as in the single linker case except there are some additional considerations.

(1) There may be a plurality of linkers for each of the two (or more) gaps to be closed. One must consider all combinations of each of the linkers for gap A with each of the linkers for gap B.

(2) One must consider the interactions between linkers.

As one must consider combinations of linkers, the ranking of individual linkers is used to cut down to a small number of very promising linkers for each gap. If one has only three candidates for each gap, there are nine possible constructs.

The process of examining interactions between linkers and discarding poor candidates can be automated by applying the rules discussed above.

V. Parallel Processing Embodiment

Figure 19 shows in block diagram form the parallel processing approach that can be utilized in the present invention.

As shown in Figure 19, a friendly serial processor 1902 is connected by a first bus 1904 to a plurality of data storage devices and input devices. Specifically, and only for purposes of illustration, a tape input stage 1906 is connected to bus 1904 so as to read into the system the parameters of the protein data base that is used. A high storage disk drive system 1908 (having, for example, 5 gigabits of storage) is also connected to bus 1904. Operationally, for even larger storage capabilities, an optical disk storage stage 1910 of conventional design can be connected to bus 1904.

The goal of the hypercube 1912 that is connected to the friendly serial processor 1902 via a bi-directional bus 1914 is twofold: to perform searching faster, and to throw out candidates more automatically.

The hypercube 1912, having for example, 2^{10} to 2^{16} nodes provides for parallel processing. There are computers currently available which have up to 1,024 computing nodes. Thus each node would need to hold only about 1400 candidate linkers and local memory of available machines would be sufficient. This is the concept of the hypercube 1912. Using the hypercube parallel processing approach, the protein data base can be divided into as many parts as there are computing nodes. Each node is assigned to a particular known protein structure.

The geometry of the gap that has to be bridged by a linker is sent by the friendly serial processor 1902 via bus 1914 to the hypercube stage 1912. Each of the nodes in the hypercube 1912 then processes the geometrical parameters with respect to the particular candidate linker to which it is assigned. Thus, all of the candidates can be examined in a parallel fashion, as opposed to the serial fashion that is done in the present mode of the present invention. This results in much faster location (the inventors believe that the processing speed can be brought down from 6 hours to 3 minutes using conventional technology) in locating the candidates that can be evaluated by the second step 304 of the present invention.

Another advantage for the parallel processing embodiment is that it will provide sufficient speed to allow candidates to be thrown out more automatically. This would be achieved using molecular dynamics and energy minimization. While this could be done currently on serial processing computers (of the super computer variety such as those manufactured by Cray

and Cyber) the parallel processing approach will perform the molecular dynamics and energy minimization much faster and cheaper than using the super computing approach.

In particular, hypercube computers exist which have inexpensive computing nodes which compare very favorably to supercomputers for scalar arithmetic. Molecular dynamics and energy minimization are only partly vectorizable because the potential functions used have numerous data-dependent branches.

VI. Preparation and Expression of Genetic Sequences, and Uses.

The polypeptide sequences generated by the methods described herein, give rise by application of the genetic code, to genetic sequences coding therefor. Given the degeneracy of the code, however, there are in many instances multiple possible codons for any one amino acid. Therefore, codon usage rules, which are also well understood by those of skill in the art, can be utilized for the preparation of optimized genetic sequences for coding in any desired organism. (See, for example, Ikemura, J. Mol. Biol. 151:389-409 (1981)).

Generally, it is possible to utilize the cDNA sequences obtained from the light and heavy chains of the variable region of the original antibody as a starting point. These sequences can then be joined by means of genetic linkers coding for the peptide linker candidates elucidated by the methods of the invention. The genetic sequence can be entirely synthesized de novo or fragments of cDNA can be linked together with the synthetic linkers, as described.

A large source of hybridomas and their corresponding monoclonal antibodies are available for the preparation of sequences coding for the H and L chains of the variable region. As indicated previously, it is well known that most "variable" regions of antibodies of a given class are in fact quite constant in their three dimensional folding pattern, except for certain specific hypervariable loops. Thus, in order to choose and determine the specific binding specificity of the single chain binding protein of the invention it becomes necessary only to define the protein sequence (and thus the underlying genetic sequence) of the hypervariable region. The hypervariable region will vary from binding molecule to molecule, but the remaining domains of the variable region will remain constant for a given class of antibody.

Source mRNA can be obtained from a wide range of hybridomas. See for example the catalogue ATCC Cell Lines and Hybridomas, December 1984, American Type Culture Collection, 20309 Parklawn Drive, Rockville, Maryland 20852, U.S.A., at pages 5-9. Hybridomas secreting monoclonal antibodies reactive with a wide variety of antigens are listed therein, are available from the collection, and usable in the invention. Of particular interest are hybridomas secreting antibodies which are reactive with viral antigens, tumor associated antigens, lymphocyte antigens, and the like. These cell lines and others of similar nature can be utilized to copy mRNA coding for the variable region or determine amino acid sequence from the monoclonal antibody itself. The specificity of the antibody to be engineered will be determined by the original se-

lection process. The class of antibody can be determined by criteria known to those skilled in the art. If the class is one for which there is a three-dimensional structure, one needs only to replace the sequences of the hyper-variable regions (or complementary determining regions). The replacement sequences will be derived from either the amino acid sequence or the nucleotide sequence of DNA copies of the mRNA.

It is to be specifically noted that it is not necessary to crystallize and determine the 3-D structure of each variable region prior to applying the method of the invention. As only the hypervariable loops change drastically from variable region to variable region (the remainder being constant in the 3-D structure of the variable region of antibodies of a given class), it is possible to generate many single chain 3-D structures from structures already known or to be determined for each class of antibody.

For example, linkers generated in the Examples in this application (e.g., TRY40, TRY61 or TRY59, see below) are for Fv regions of antibodies of the IgA class. They can be used universally for any antibody, having any desired specificity, especially if the antibody is of the IgA class.

Expression vehicles for production of the molecules of the invention include plasmids or other vectors. In general, such vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with the host. The vector ordinarily carries a replicon site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. For example, E. coli is readily transformed

using pBR322, a plasmid derived from an E. coli species. pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides easy means for identifying transformed cells. The pBR322 plasmid or other microbial plasmids must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the beta lactamase, lactose promoter systems, lambda phage promoters, and the tryptophan promoter systems. While these are the most commonly used, other microbial promoters have been discovered and can be utilized.

For example, a genetic construct for a single chain binding protein can be placed under the control of the leftward promoter of bacteriophage lambda. This promoter is one of the strongest known promoters which can be controlled. Control is exerted by the lambda repressor, and adjacent restriction sites are known.

The expression of the single chain antibody can also be placed under control of other regulatory sequences which may be homologous to the organism in its untransformed state. For example, lactose dependent E. coli chromosomal DNA comprises a lactose or lac operon which mediates lactose utilization by elaborating the enzyme beta-galactosidase. The lac control elements may be obtained from bacteriophage lambda plac5, which is infective for E. coli. The lac promoter-operator system can be induced by IPTG.

Other promoter/operator systems or portions thereof can be employed as well. For example, colicin E1,

galactose, alkaline phosphatase, tryptophan, xylose, tac, and the like can be used.

Of particular interest is the use of the O_L/P_R hybrid lambda promoter (see for example U.S. patent application Serial Number 534,982 filed September 3, 1983, and herein incorporated by reference).

Other preferred hosts are mammalian cells, grown in vitro in tissue culture, or in vivo in animals. Mammalian cells provide post translational modifications to immunoglobulin protein molecules including correct folding or glycosylation at correct sites.

Mammalian cells which may be useful as hosts include cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/0-AG14 or the myeloma P3x63Sg8, and their derivatives.

Several possible vector systems are available for the expression of cloned single chain binding proteins in mammalian cells. One class of vectors utilizes DNA elements which provide autonomously replicating extrachromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, or SV40 virus. A second class of vectors relies upon the integration of the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing drug resistance genes such as E. coli GPT or Tn5neo. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding

protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., Mol. Cel. Biol., 3:280 (1983), and others.

Another preferred host is yeast. Yeast provides substantial advantages in that it can also carry out post translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products, and secretes peptides bearing leader sequences (i.e., pre-peptides).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in mediums rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Once the strain carrying the single chain building molecule gene has been constructed, the same can also be subjected to mutagenesis techniques using, chemical agents or radiation, as is well known in the art. From the colonies thus obtained, it is possible to search for those producing binding molecules with increased binding affinity. In fact, if the first lin-

ker designed with the aid of the computer fails to produce an active molecule, the host strain containing the same can be mutagenized. Mutant molecules capable of binding antigen can then be screened by means of a routine assay.

The expressed and refolded single chain binding proteins of the invention can be labelled with detectable labels such as radioactive atoms, enzymes, biotin/avidin labels, chromophores, chemiluminescent labels, and the like for carrying out standard immunodiagnostic procedures. These procedures include competitive and immunometric (or sandwich) assays. These assays can be utilized for the detection of antigens in diagnostic samples. In competitive and/or sandwich assays, the binding proteins of the invention can also be immobilized on such insoluble solid phases as beads, test tubes, or other polymeric materials.

For imaging procedures, the binding molecules of the invention can be labelled with opacifying agents, such as NMR contrasting agents or X-ray contrasting agents. Methods of binding, labelling or imaging agents to proteins as well as binding the proteins to insoluble solid phases are well known in the art. The refolded protein can also be used for therapy when labelled or coupled to enzymes or toxins, and for purification of products, especially those produced by the biotechnology industry. The proteins can also be used in biosensors.

Having now generally described this invention the same will be better understood by reference to certain specific examples which are included for purposes of illustration and are not intended to be limiting unless otherwise specified.

EXAMPLES

In these experiments, the basic Fv 3-D structure used for the computer assisted design was that of the anti-phosphoryl choline myeloma antibody of the IgA class, MCPC-603. The X-ray structure of this antibody is publicly available from the Brookhaven data base.

The starting material for these examples was monoclonal antibody cell line 3C2 which produced a mouse anti-bovine growth hormone (BGH). This antibody is an IgG_1 with a gamma 1 heavy chain and kappa light chain. cDNA's for the heavy and light chain sequences were cloned and the DNA sequence determined. The nucleotide sequences and the translation of these sequences for the mature heavy and mature light chains are shown in Figures 21 and 22 respectively.

Plasmids which contain just the variable region of the heavy and light chain sequences were prepared. A ClaI site and an ATG initiation codon (ATCGATG) were introduced before the first codon of the mature sequences by site directed mutagenesis. A HindIII site and termination codon (TAAGCTT) were introduced after the codon 123 of the heavy chain and the codon 109 of the light chain. The plasmid containing the V_H sequences is pGX3772 and that containing the V_L is pGX3773 (Figure 23).

The examples below were constructed and produced by methods known to those skilled in the art.

EXAMPLE 1

A. Computer Design

A two-linker example (referred to as TRY 40) was designed by the following steps.

First, it was observed that light chains were much easier to make in E. coli than were heavy chains. It was thus decided to start with light chain. (In the future, one could certainly make examples which begin with heavy chain because there is a very similar contact between a turn in the heavy chain and the exit strand of the light chain.)

Refer to stereo Figure 30A, which shows the light and heavy domains of the Fv from MOPC-603 antibody; the constant domains are discarded. A line joining the alpha carbons of the light chain is above and dashed. The amino terminus of the light chain is to the back and at about 10 o'clock from the picture center and is labeled "N." At the right edge of the picture, at about 2 o'clock is an arrow showing the path toward the constant domain. Below the light chain is a line joining the alpha carbons of the heavy chain. The amino terminus of the heavy chain is toward the viewer at about 7 o'clock and is also labeled "N." At about 4:30, one sees an arrow showing the heavy chain path to its constant domain.

The antigen-binding site is to the left, about 9 o'clock and between the two loops which project to the right above (light chain) and below (heavy chain).

In addition to the alpha carbon traces, there are three segments in which all non-hydrogen atoms have been drawn. These strands are roughly parallel and from upper right to lower left. They are

(a) Proline 46 to Proline 50 of the light chain.

(b) Valine 111 to Glycine 113 of the heavy chain.

(c) Glutamic acid 1 to glycine 10 of the heavy chain.

The contact between tryptophan 112 of the heavy chain and proline 50 of the light chain seems very favorable. Thus it was decided that these two residues should be conserved. Several linkers were sought and found which would join a residue at or following Tryptophan 112 (heavy) to a residue at or following Proline 50 (light). Stereo figure 30B shows the region around TRP 112H in more detail. The letter "r" stands between the side-chain of TRP 112H and PRO 50L; it was wished to conserve this contact. The letter "q" labels the carboxy terminal strand which leads towards the constant domain. It is from this strand that a linker will be found which will connect to PRO 50L.

Once a linker is selected to connect 112H to 50L, one needs a linker to get from the first segment of the light chain into the beginning portion of the heavy chain. Note that PRO 46L turns the chain toward PRO 50L. This turning seemed very useful, so it was decided to keep PRO 46L. Thus the second linker had to begin after 46L and before 50L, in the stretch marked "s." A search for linkers was done beginning on any of the residues 46L, 47L, or 48L. Linkers beginning on residue 49L were not considered because the chain has already turned toward 50L and away from the amino terminal of the heavy chain. Linkers were sought which ended on any of the residues 1H to 10H.

Figure 30C shows the linked structure in detail. After TRP 112H and GLY 113H, was introduced the sequence PRO-GLY-SER, and then comes PRO 50L. A computer program was used to look for short contacts between atoms in the linker and atoms in the retained part of the Fv. There is one short contact between the beta carbon of the SER and PRO 50L, but small movements would relieve that. This first linker runs from the point labeled "x" to the point labeled "y." The second linker runs from "v" to "w." Note that most of the hydrophobic residues (ILE and VAL) are inside. There is a PHE on the outside. In addition, the two lysine residues and the asparagine residue are exposed to solvent as they ought to be. Figure 30D shows the overall molecule linked into a single chain.

B. Genetic Constructs

These constructs were prepared and the plasmids containing them using E. coli. hosts. Once constructed, the sequences can be inserted into whichever expression vehicle used in the organism of choice.

The first construction was TRY40 (the two-linker construction) which produces a protein with the following sequence:

Met-[L-chain 1-41]-Ile-Ala-Lys-Ala-Phe-Lys-Asn-[H-chain 8-105]-Pro-Gly-Ser-[L-chain 45-109]. The nucleotide sequence and its translation are seen in Figure 24. The hypervariable regions in TRY40 (as in TRY61 59 and 104B, see below) correspond, as indicated, to an IgG1 anti BGH antibody, even though the 3-D analysis was done on the Fv region of MCPC-603 antibody, having a different specificity, (anti phosphoryl choline) but having a similar framework in the variable region.

The antibody sequences in the plasmids pGX3772 and pGX3773 were joined to give the sequence of TRY40 in the following manner. The plasmids used contained an M13 bacteriophage origin of DNA replication. When hosts containing these plasmids are superinfected with bacteriophage M13 two types of progeny are produced, one containing the single-strand genome and the other containing a specific circular single-strand of the plasmid DNA. This DNA provided template for the oligonucleotide directed site specific mutagenesis experiments that follow. Template DNA was prepared from the two plasmids. An EcoRI site was introduced before codon 8 of the V_H sequence in pGX3772, by site directed mutagenesis, producing pGX3772'. Template from this construction was prepared and an XbaI site was introduced after codon 105 of the V_H sequence producing pGX3772''.

An EcoRI and an XbaI site were introduced into pGX3773 between codons 41 and 45 of the V_L sequence by site directed mutagenesis producing pGX3773'.

To begin the assembly of the linker sequences plasmid pGX3773' (V_L) DNA was cleaved with EcoRI and XbaI and treated with calf alkaline phosphatase. This DNA was ligated to the EcoRI to XbaI fragment purified from plasmid pGX3772''(V_H) which had been cleaved with the two restriction enzymes. The resulting plasmid pGX3774, contained the light and heavy chain sequences in the correct order linked by the EcoRI and XbaI restriction sites. To insert the correct linker sequences in frame, pGX3774 template DNA was prepared. The EcoRI junction was removed and the linker coding for the -Ile-Ala-Lys-Ala-Phe-Lys-Asn- inserted by site

directed mutagenesis, producing plasmid pGX3774'. Template DNA was prepared from this construction and the XbaI site corrected and the linker coding for -Pro-Gly-Ser- inserted by site directed mutagenesis producing plasmid pGX3775. The sequence was found to be correct as listed in Figure 24 by DNA sequencing.

In order to express the single-chain polypeptide, the sequence as a ClaI to HindIII fragment was inserted into a vector pGX3703. This placed the sequence under the control of the O_L/P_R hybrid lambda promoter (U.S. Patent Application 534,982, Sept. 23, 1983). The expression plasmid is pGX3776 (Figure 25). The plasmid pGX3776 was transformed into a host containing a heat sensitive lambda phage repressor; when grown at 30°C the synthesis of the TRY40 protein is repressed. Synthesis was induced by raising the temperature to 42°C, and incubating for 8-16 hours. The protein was produced at 7.2% of total cell protein, as estimated on polyacrylamide gel electropherograms stained with Coomassie blue.

EXAMPLE 2

A. Computer Design

A one-linker example (referred to as TRY 61) was designed by the following steps.

Refer to stereo Figure 31A which shows the light and heavy domains of the Fv; the constant domains are discarded. A line joining the alpha carbons of the light chain is dashed. The amino terminus of the light chain is to the back and at about the center of the picture and is labeled "N." At the right edge of the picture, at about 2 o'clock is an arrow showing

the path toward the constant domain of the light chain. Below the light chain is a line joining the alpha carbons of the heavy chain. The amino terminus of the heavy chain is toward the viewer at about 9 o'clock and is also labeled "N". At about 4:30, one sees an arrow showing the heavy chain path to its constant domain.

In addition to the alpha carbon traces, there are two segments in which all non-hydrogen atoms have been drawn. These segments are the last few residues in the light chain and the first ten in the heavy chain. Linkers were sought between all pairs of these residues, but only a few were found because these regions are widely separated.

Figure 31B shows the linker in place. Note that the molecule now proceeds from the amino terminal of the light chain to the carboxy terminal strand of the heavy chain. Note also that the antigen-binding region is to the left, on the other side of the molecule from the linker.

B. Genetic Constructs

The sequence of TRY61 (a single-linker embodiment) is Met-[L-chain 1-104]-Val-Arg-Gly-Ser-Pro-Ala-Ile-Asn-Val-Ala-Val-His-Val-Phe-[H-chain 7-123]. The nucleotide sequence and its translation are shown in Figure 26.

To construct TRY61, plasmid pGX3772' DNA was cleaved with ClaI and EcoRI and treated with calf alkaline phosphatase. This DNA was ligated with the ClaI to HindIII fragment from pGX3773 and two oligonucleotides which code for the linker sequence and have HindIII and EcoRI ends, so that the linker can

only be ligated in the correct orientation. The resulting plasmid, pGX3777, was used to prepare template DNA. This DNA was used for site directed mutagenesis to remove the HindIII site inside the antibody sequences. The correct construction, pGX3777', was used to make template DNA for a site directed mutagenesis to remove the EcoRI site. The ClaI to HindIII fragment from the final construction, pGX3778, containing the TRY61 coding sequence was confirmed by DNA sequencing. The ClaI to HindIII was inserted into the pGX3703 expression vector. This plasmid is called pGX4904 (Figure 27). This plasmid was transformed into an E. coli host. The strain containing this plasmid has been induced, and the single chain protein produced as >2% of total cell protein.

EXAMPLE 3

A. Computer Design

A one-linker example (referred to as TRY 59) was designed by the following steps.

Refer to stereo Figure 32A which shows the light and heavy domains of the Fv; the constant domains are discarded. A line joining the alpha carbons of the light chain is above and dashed. The amino terminus of the light chain is to the back and at about 10 o'clock from the center of the picture and is labeled "N". At the right edge of the picture, at about 2 o'clock is an arrow showing the path toward the constant domain of the light chain. Below the light chain is a line joining the alpha carbons of the heavy chain. The amino terminus of the heavy chain is toward the viewer at about 8 o'clock and is also labeled

"N". At about 4:30, one sees an arrow showing the heavy chain path to its constant domain.

In addition to the alpha carbon traces, there are two segments in which all non-hydrogen atoms have been drawn. These segments are the last few residues in the light chain and the first ten in the heavy chain. Linkers we sought between all pairs of these residues, but only a few were found because these regions are widely separated.

Figure 32B shows the linker in place. Note that the molecule now proceeds from the amino terminal of the light chain to the carboxy terminal strand of the heavy chain. Note also that the antigen-binding region is to the left, on the other side of the molecule from the linker.

The choice of end points in TRY59 is very similar to TRY61. Linkers of this length are rare. The tension between wanting short linkers that fit very well and which could be found for the two-linker case (TRY40) and the desire to have only one linker, (which is more likely to fold correctly) is evident in the acceptance of TRY59. The linker runs from the point marked "A" in Figure 32B to the point marked "J." After five residues, the linker becomes helical. At the point marked "x," however, the side-chain of an ILE residue collides with part of the light chain. Accordingly, that residue was converted to GLY in the actual construction.

B. Genetic Constructs

The sequence of TRY59 (the single linker construction) is Met-[L-chain 1-105]-Lys-Glu-Ser-Gly-Ser-Val-Ser-Ser-Glu-Gln-Leu-Ala-Gln-Phe-Arg-Ser-Leu-Asp-[H-

chain 2-123]. The nucleotide sequence coding for this amino acid sequence and its translation is shown in Figure 28. The BglI to HindIII fragment (read clockwise) from plasmid pGX3773 containing the V_L sequence and the ClaI to BglI fragment (clockwise) from pGX3772 has been ligated with two oligonucleotides which form a fragment containing the linker sequence for TRY59 and have ClaI and HindIII ends. The ClaI and HindIII junctions within this plasmid are corrected by two successive site directed mutageneses to yield the correct construction. The ClaI to HindIII fragment from this plasmid is inserted into the O_L/P_R expression vector as in Examples 1 and 2. The resulting plasmid, pGX4908 (Figure 29) is transformed into an E. coli host. This strain is induced to produce the protein coded by the sequence in Figure 28 (TRY59).

Example 4

A. Computer Design

In this design an alternative method of choosing a linker to connect the light and heavy variable regions was used. A helical segment from human hemoglobin was chosen to span the major distance between the carboxy terminus of the variable light chain and the amino terminus of the variable heavy chain. This alpha helix from human hemoglobin was positioned at the rear of the F_v model using the computer graphics system. Care was taken to position the helix with its ends near the respective amino and carboxyl termini of the heavy and light chains. Care was also taken to place hydrophobic side chains in toward the F_v and hydro-

philic side chains toward the solvent. The connections between the ends of the variable regions and the hemoglobin helix were selected by the previously described computer method (EXAMPLE 1-3).

B. Genetic Constructs

The sequence of TRY104b (a single linker construction) is Met-[L-chain 1-106]-Ala-Glu-Gly-Thr-[(Hemoglobin helix)Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-]Met-Thr-[H-chain 3-123]. The nucleotide sequence coding for this amino acid sequence and its translation is shown in Figure 33. The BglI to HindIII fragment (read clockwise) from plasmid pGX3773 containing the V_L sequence and the ClaI to BglI fragment (clockwise) from pGX3772 has been ligated with two oligonucleotides which form a fragment containing the linker sequence for TRY104b and have ClaI and HindIII ends. The ClaI and HindIII junctions within this plasmid are corrected by two successive site directed mutageneses to yield the correct construction. The ClaI to HindIII fragment from this plasmid is inserted into the O_L/P_R expression vector as in Examples 1-3. The resulting plasmid, pGX4910 (Figure 34) is transformed into an E. coli host. This strain is induced to produce the protein coded by the sequence in Figure 33 (TRY104b).

EXAMPLE 5

Purification of the Proteins

The single-chain antigen binding proteins from TRY40, TRY61, TRY59 and TRY104b are insoluble, and cells induced to produce these proteins show refractile bodies called inclusions upon microscopic exami-

nation. Induced cells were collected by centrifugation. The wet pellet was frozen on dry ice, then stored at -20°C. The frozen pellet was suspended in a buffer and washed in the same buffer, and subsequently the cells were suspended in the same buffer. The cells were broken by passage through a French pressure cell, and the inclusion bodies containing the single-chain antigen binding protein (SCA) were purified by repeated centrifugation and washing. The pellet was solubilized in guanidine-HCl, and reduced with 2-mercaptoethanol. The solubilized material was passed through a gel filtration column, i.e., Sephacryl™ S-300. Other methods such as ion exchange could be used.

EXAMPLE 6

Folding of the Proteins

Purified material was dialyzed against water, and the precipitate protein collected by centrifugation. The protein was solubilized in urea and reduced with 2-mercaptoethanol. This denatured and solubilized material was dialyzed against a buffer containing salt and reducing agents to establish the redox potential to form the intra domain (one each for the light and heavy chain variable region sequences) disulfide bridges (Saxena and Wetlanfer, Biochem 9:5015-5023 (1970)). The folded protein was assayed for BGH binding activity.

The TRY59 protein used in competition experiments was solubilized and renatured directly from inclusions. This material was subsequently purified by affinity to BGH-Sepharose.

EXAMPLE 7

Binding Assay

BGH was immobilized on nitrocellulose strips along with non-specific proteins such as bovine serum albumin or lysozymes. Further non-specific protein binding was blocked with an immunologically inert protein, for example gelatin. Folded SCA was tested for its ability to bind to BGH. The SCA was detected by a rabbit anti-L chain (of the monoclonal) anti-serum. The rabbit antibodies were reacted with goat anti-rabbit IgG coupled to peroxidase. The strips were reacted with chemicals which react with the peroxidase to give a color reaction if the peroxidase is present.

Figure 35 shows the result of this spot assay for TRY61 (strip 1) and TRY40 (strip 2). Strip 3 was stained with amido black to show the presence of all three proteins. The other proteins, TRY59, TRY104b gave similar results in the spot assay. A competition assay with the SCA competing with the monoclonal can be used as well. The results of competing the F_{ab} of 3C2 monoclonal with 1 and 10 ug of TRY59 protein which had been affinity purified are shown in Figure 36 (▲ F_{ab} alone, ■ F_{ab} + 1 ug TRY59, and ● F_{ab} + 10 ug TRY59). The affinity estimated from the Ic_{50} of this experiment was approximately 10^6. The data are summarized in Table 1.

Table 1

Properties of the Proteins Produced by the Single-chain Constructions

Single-chain	Number of Linkers	Spot Test	Binding to BGH Column	Ka
TRY40	2	+	-	ND
TRY59	1	+	+	$\sim 10^8$
TRY61	1	+	-	ND
TRY104B	1	+	+	ND
3C2 monoclonal	NA	+	+	$10^8 - 10$

ND = not determined
NA = not applicable

WE CLAIM:

1. A single polypeptide chain binding molecule which has binding specificity substantially similar to the binding specificity of the light and heavy chain aggregate variable region of an antibody.

2. The molecule of claim 1 which comprises two peptide linkers joining said light and heavy chains into said single chain.

3. The molecule of claim 2 which comprises in sequence:

(a) an N-terminal region derived from said light chain;
(b) a peptide linker;
(c) a peptide region derived from said heavy chain;
(d) a second peptide linker; and
(e) a C-terminal region derived from said light chain.

4. The molecule of claim 1 which comprises one peptide linker joining said light and heavy chains into said single chain.

5. The molecule of claim 4 which comprises, in sequence:

(a) an N-terminal region derived from said light chain;
(b) a peptide linker; and
(c) a C-terminal region derived from said heavy chain.

6. The molecule of claim 4 which comprises in sequence:

(a) an N-terminal region derived from said heavy chain;

(b) a peptide linker; and

(c) a C-terminal region derived from said light chain.

7. The molecule of claim 3, 5 or 6 which, prior to said N-terminal region (a), comprises a methionine residue.

8. The molecule of claim 1 which is detectably labeled.

9. The molecule of claim 1 which is in immobilized form.

10. The molecule of claim 1 which is conjugated to an imaging agent.

11. The molecule of claim 1 which is conjugated to a toxin.

12. A genetic sequence coding for the molecule of claim 1.

13. A recombinant DNA (rDNA) molecule comprising the sequence of claim 12.

14. The rDNA molecule of claim 13 which is a replicable cloning or expression vehicle.

15. The rDNA molecule of claim 14 wherein said vehicle is a plasmid.

16. A host cell transformed with the rDNA molecule of claim 13.

17. The host cell of claim 16 which is a bacterial cell, a yeast or other fungal cell or a mammalian cell line in vitro.

18. A method of producing a single polypeptide chain binding molecule which has binding specificity substantially similar to the binding specificity of the light and heavy chain aggregate variable region of an antibody, which comprises:

(a) providing a genetic sequence coding for said molecule;

(b) transforming a host cell with said sequence;

(c) expressing said sequence in said host; and

(d) recovering said molecule.

19. The method of claim 18 which further comprises purifying said recovered molecule.

20. The method of claim 18 wherein said host cell is a bacterial cell, yeast or other fungal cell, or a mammalian cell line.

21. The binding molecule produced by the method of claim 18 or 19.

22. In an immunoassay method which utilizes an antibody in labeled form, the improvement comprising using the molecule of claim 8 instead of said antibody.

23. In an immunoassay method which utilizes an antibody in immobilized form, the improvement comprising using the molecule of claim 9 instead of said antibody.

24. In the immunoassay of claim 21 or 22 wherein said immunoassay is a competitive immunoassay.

25. In the immunoassay of claim 21 or 22 wherein said immunoassay is a sandwich immunoassay.

26. In an immunotherapeutic method which utilizes an antibody conjugated to a therapeutic agent, the improvement comprising using the molecule of claim 1 instead of said antibody.

27. In a method of immunoaffinity purification which utilizes an antibody therefor, the improvement which comprises using the molecule of claim 1 instead of said antibody.

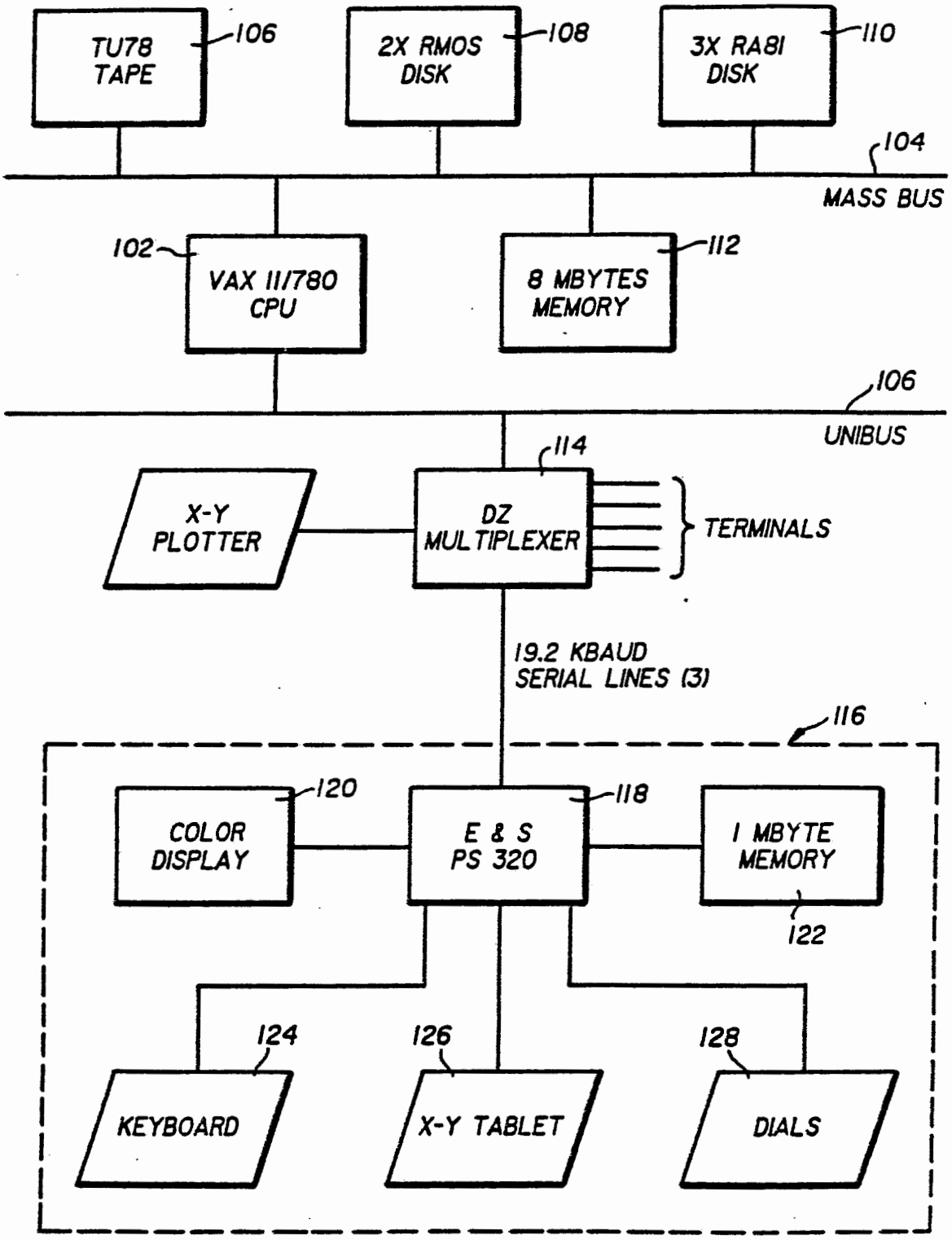


FIG. 1

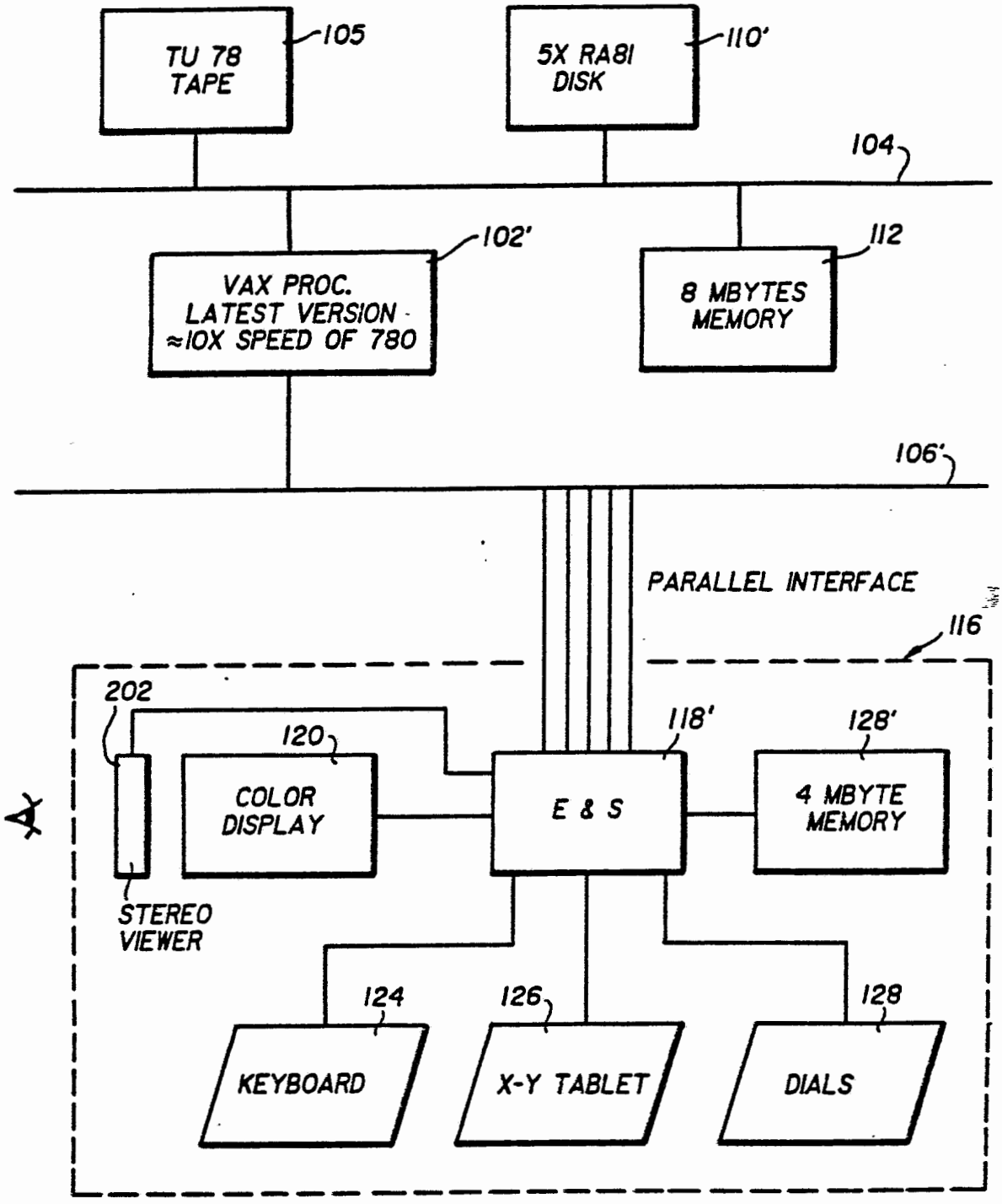


FIG. 2

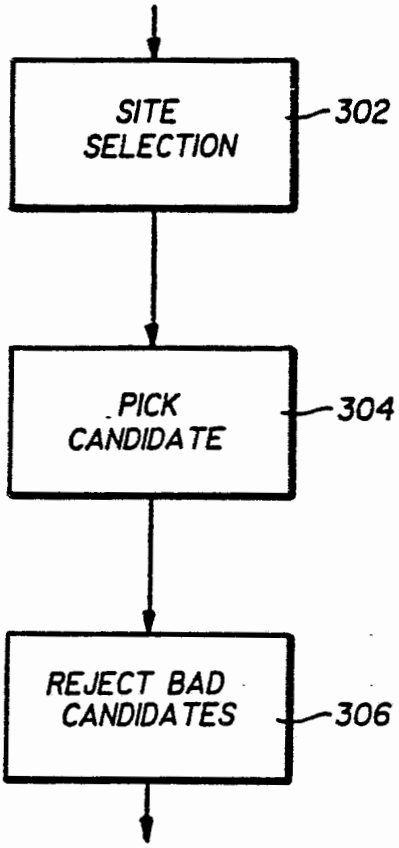


FIG. 3

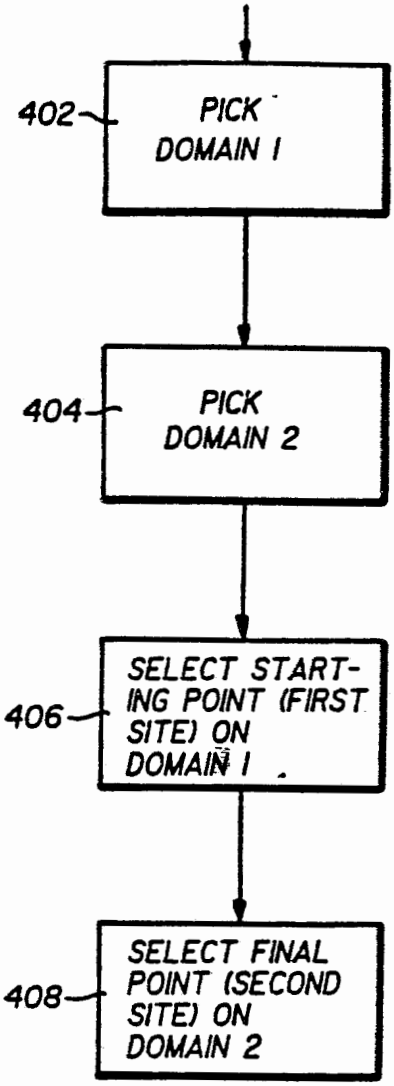


FIG. 4

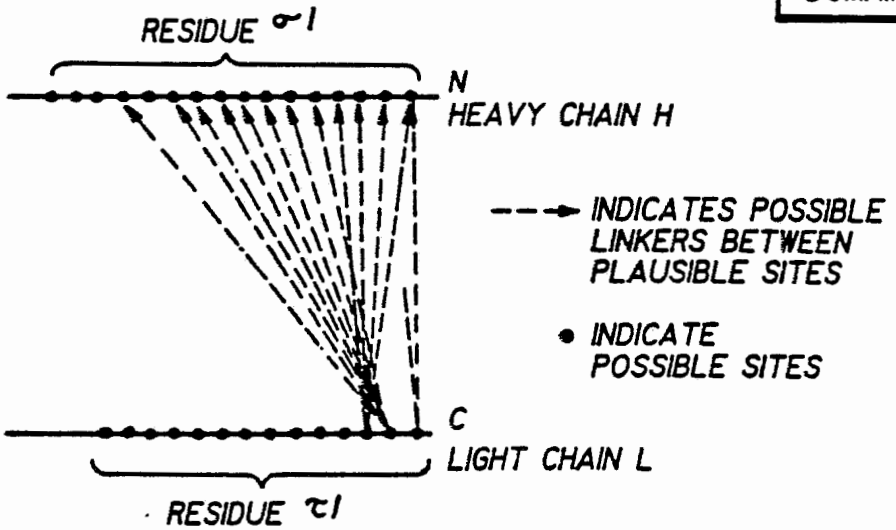


FIG. 7

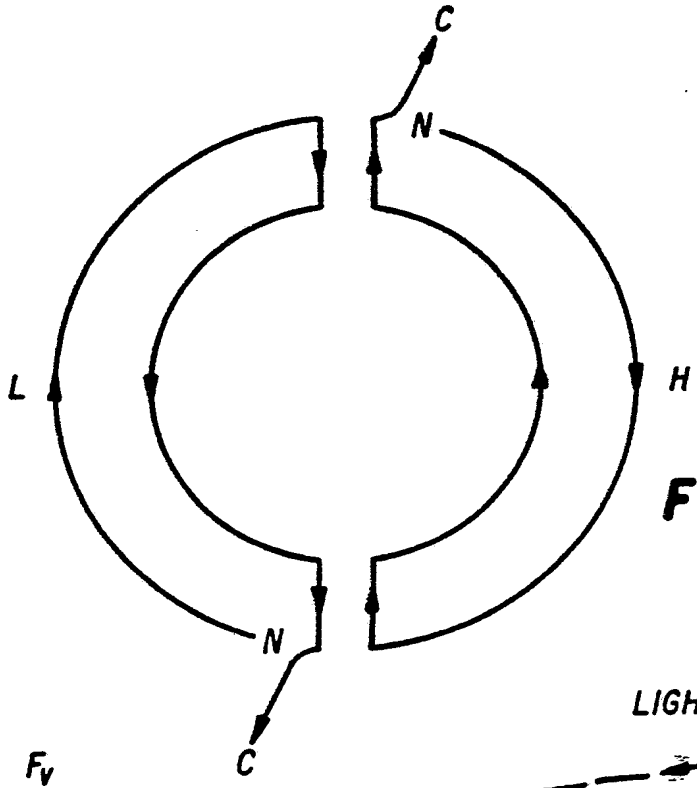


FIG. 5A

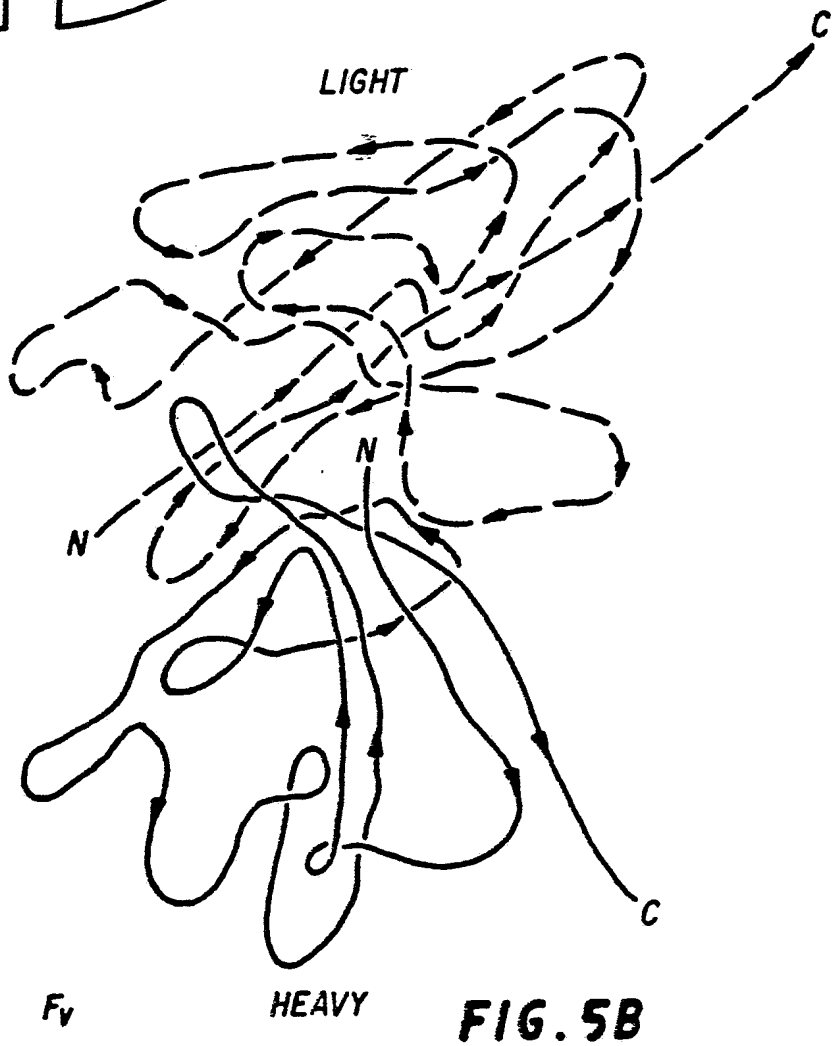


FIG. 5B

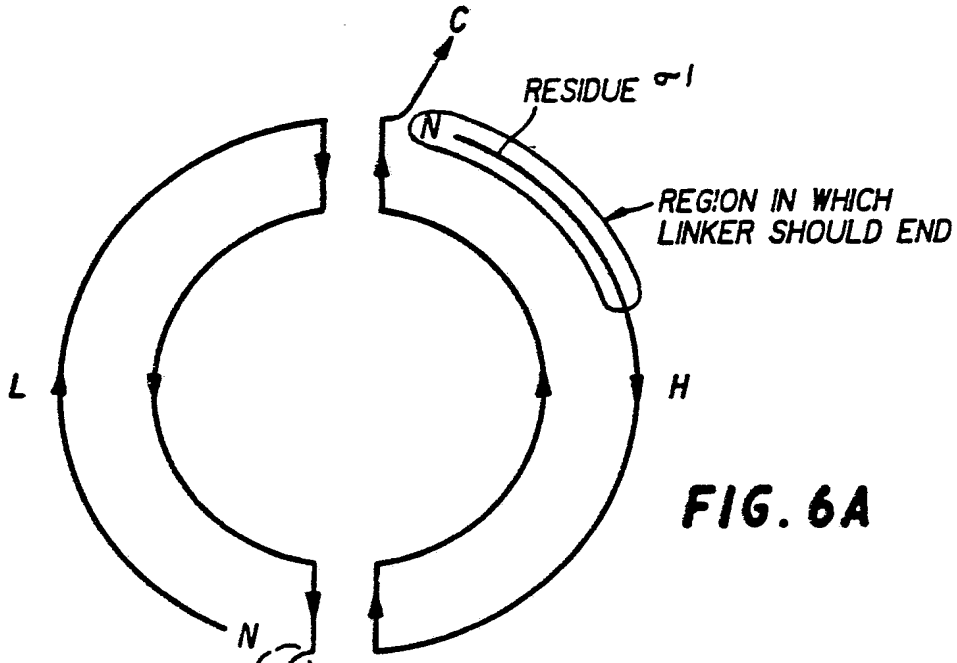


FIG. 6A

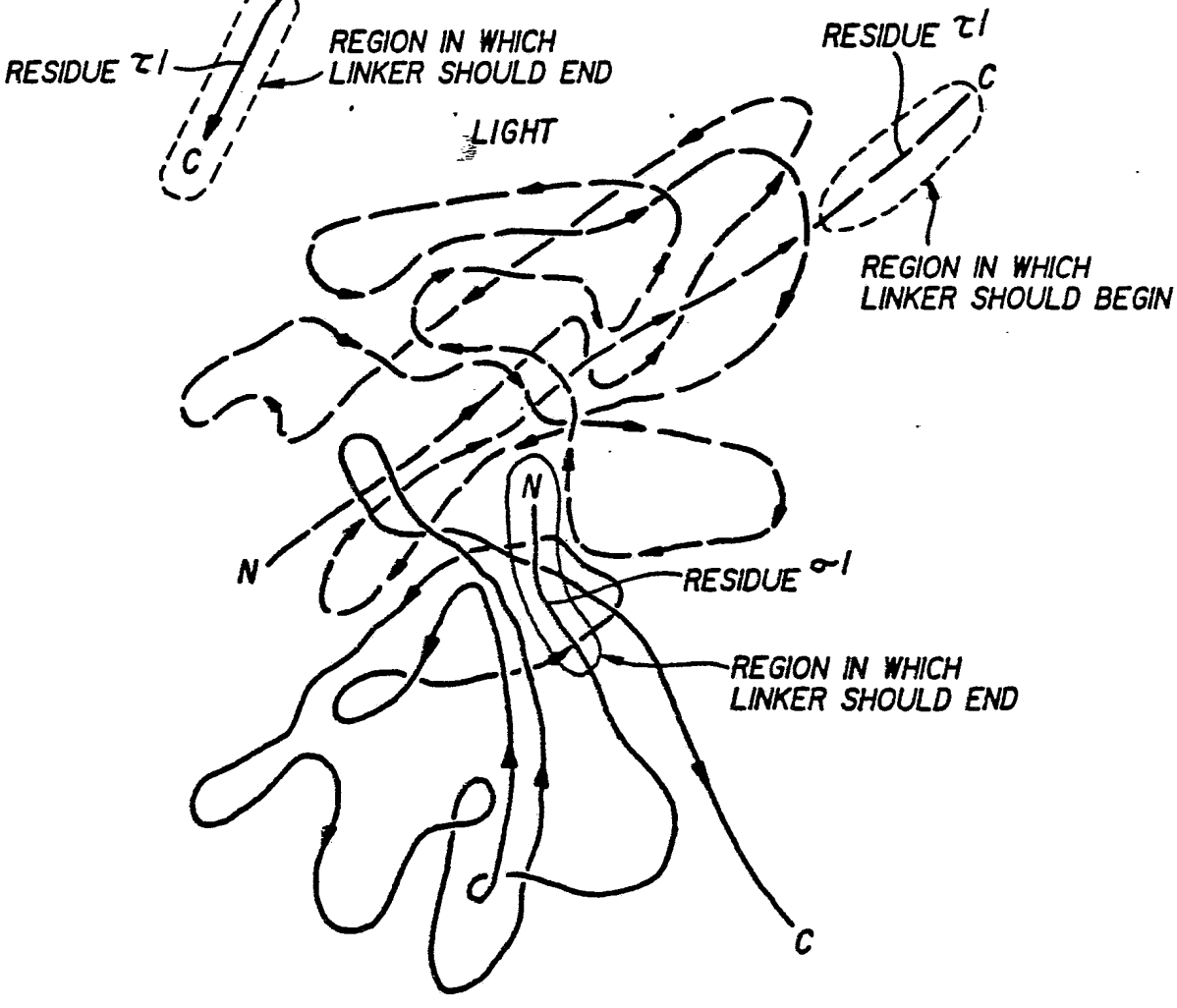


FIG. 6B

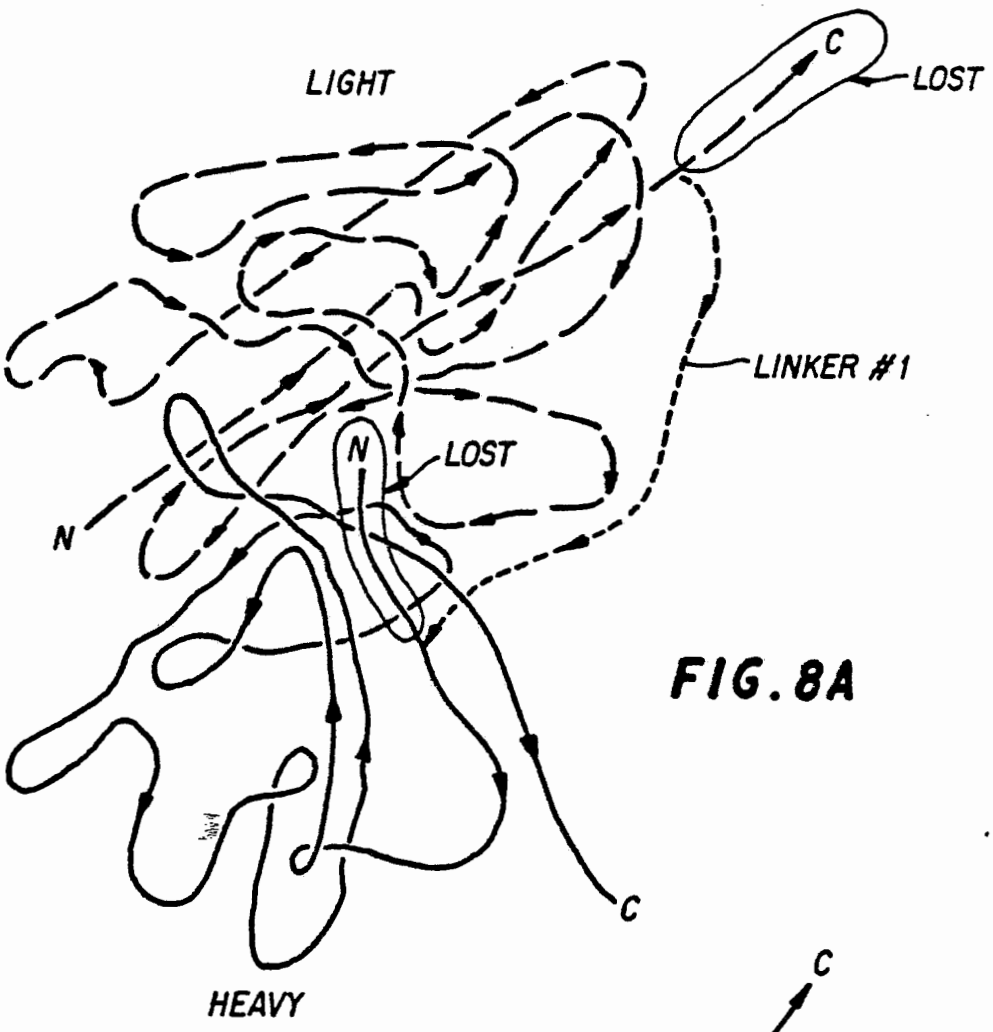


FIG. 8A

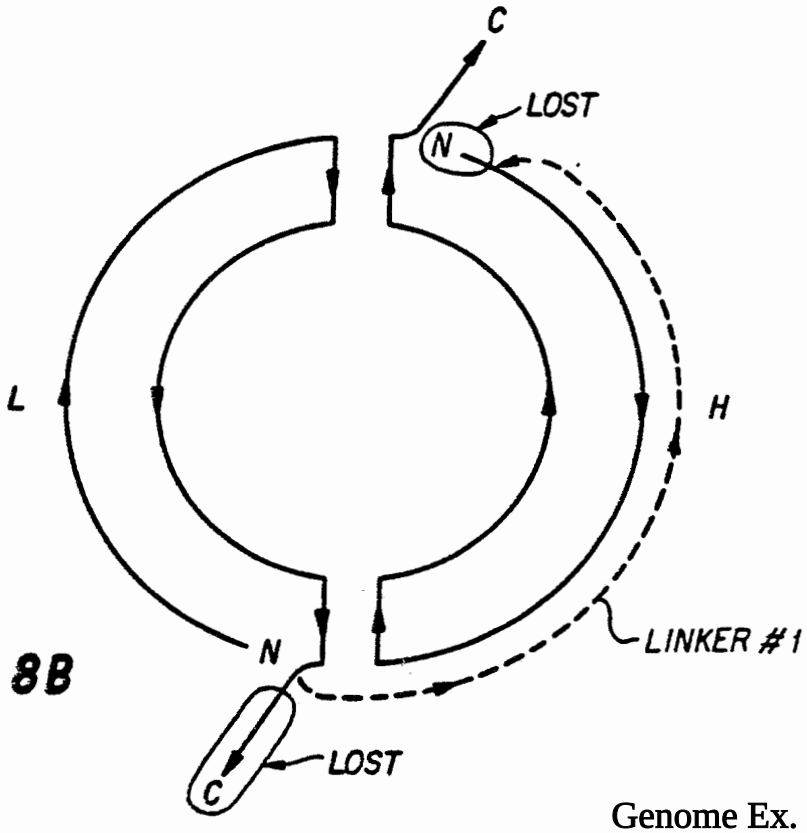


FIG. 8B

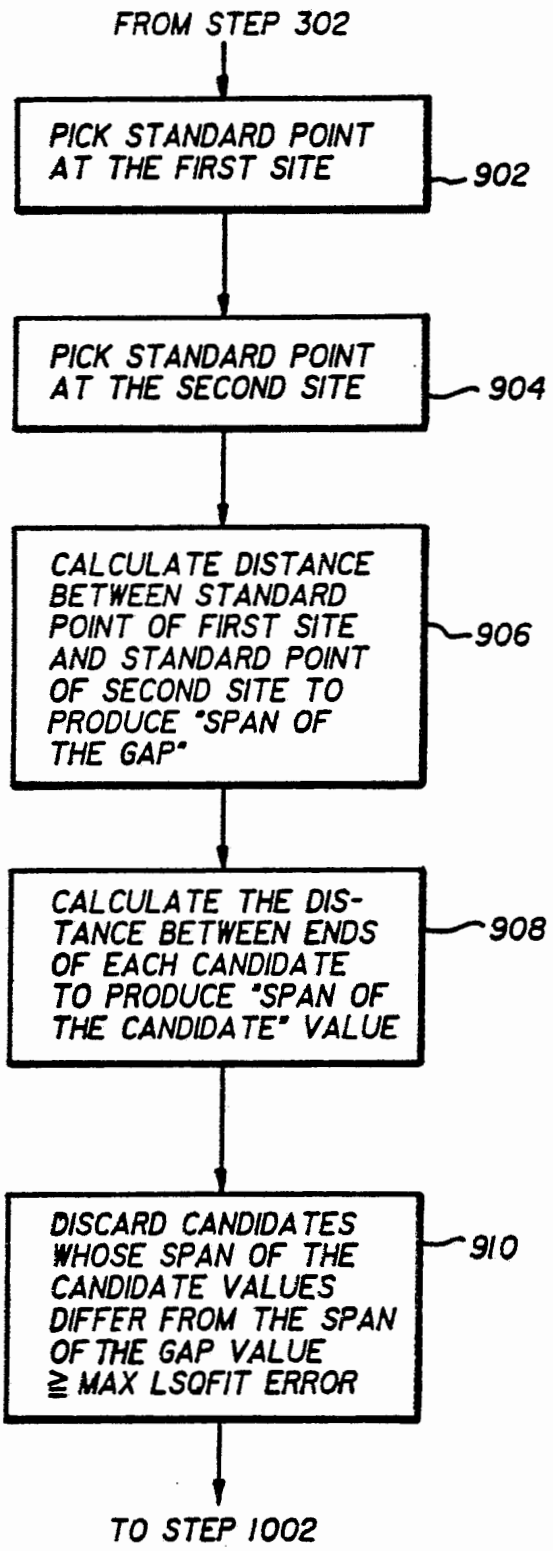


FIG. 9

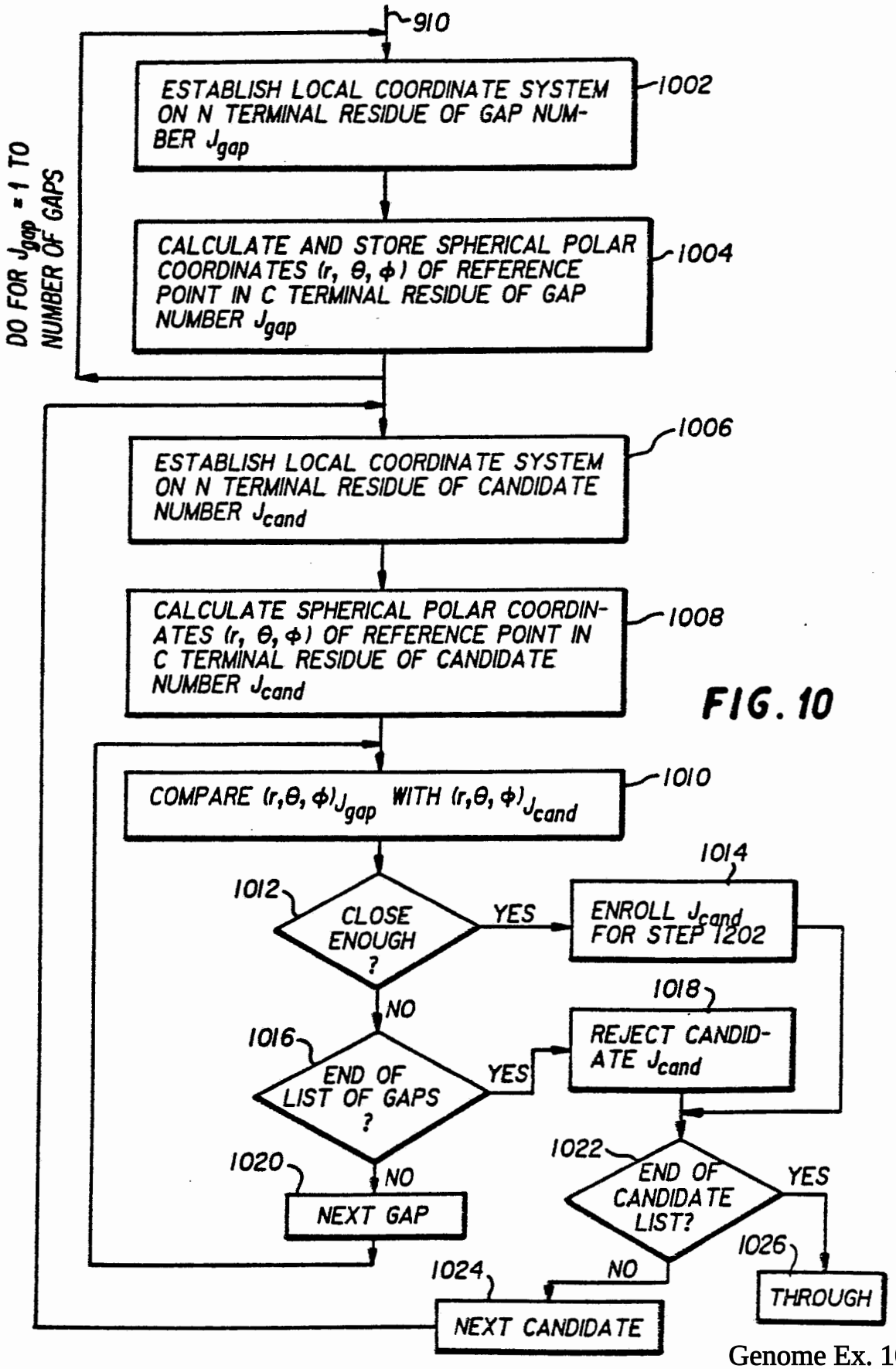


FIG. 10

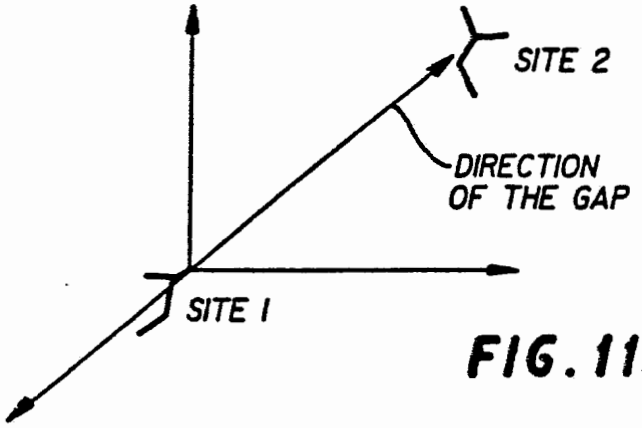


FIG. 11A

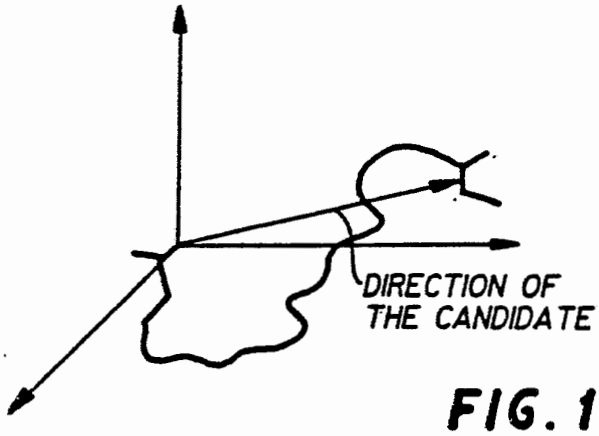


FIG. 11B

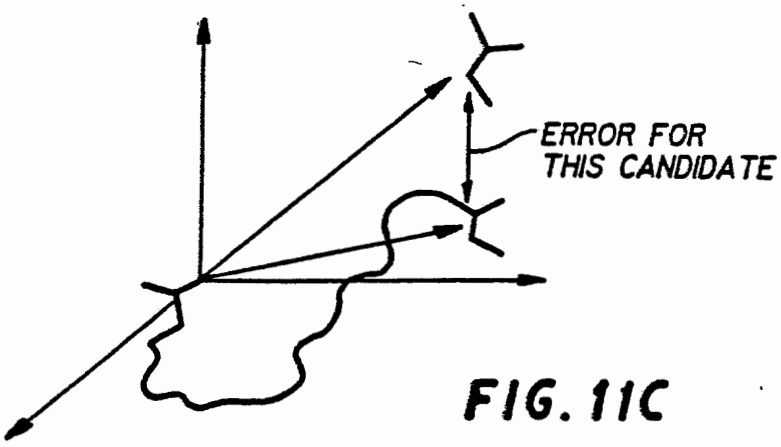


FIG. 11C

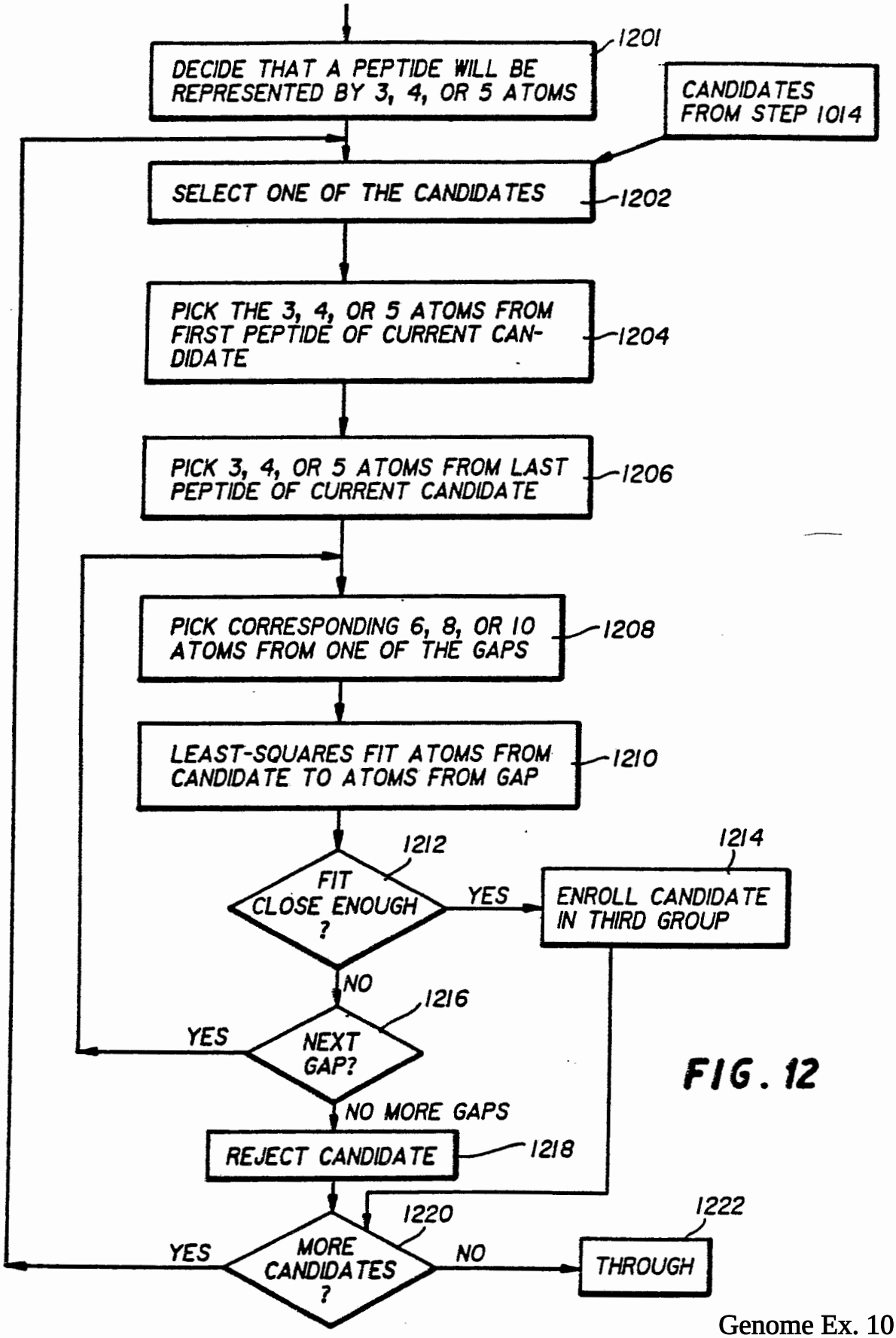


FIG. 12

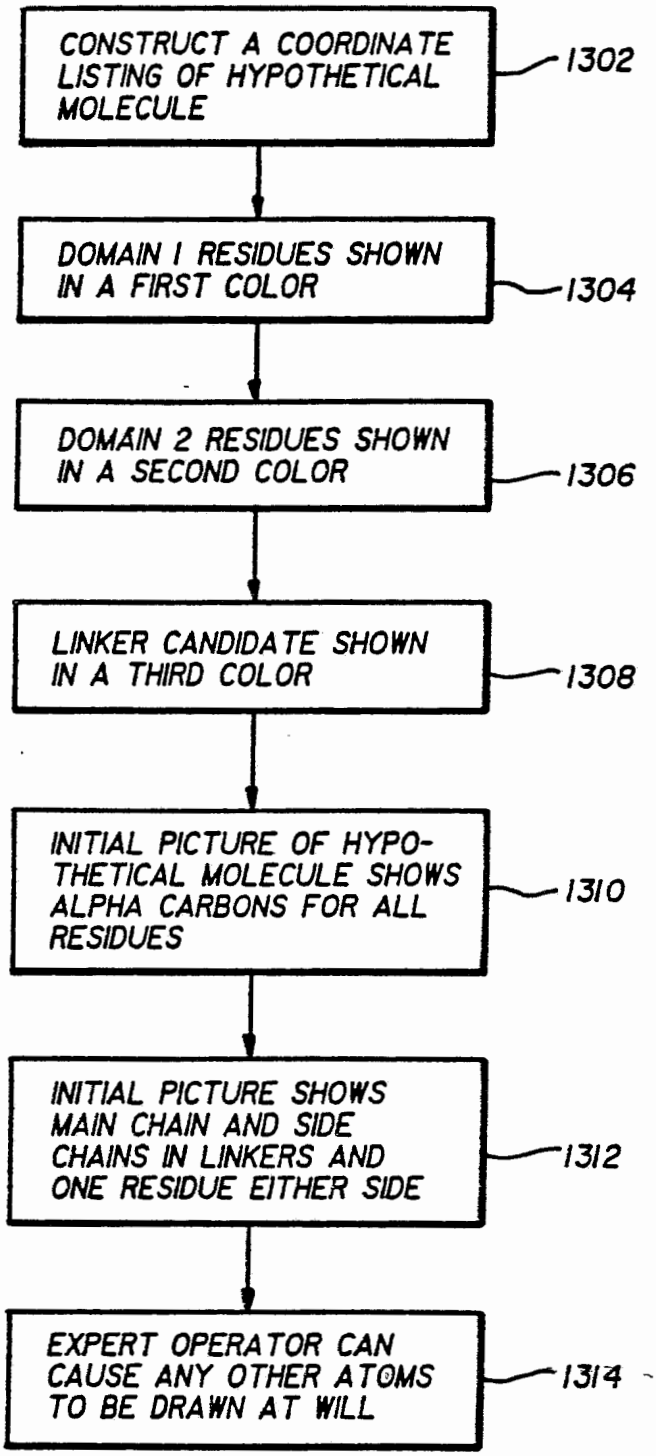

CONSTRUCT A COORDINATE LISTING OF HYPOTHETICAL MOLECULE
1302
DOMAIN I RESIDUES SHOWN IN A FIRST COLOR
1304
DOMAIN 2 RESIDUES SHOWN IN A SECOND COLOR
1306
LINKER CANDIDATE SHOWN IN A THIRD COLOR
1308
INITIAL PICTURE OF HYPOTHETICAL MOLECULE SHOWS ALPHA CARBONS FOR ALL RESIDUES
1310
INITIAL PICTURE SHOWS MAIN CHAIN AND SIDE CHAINS IN LINKERS AND ONE RESIDUE EITHER SIDE
1312
EXPERT OPERATOR CAN CAUSE ANY OTHER ATOMS TO BE DRAWN AT WILL
1314

FIG. 13

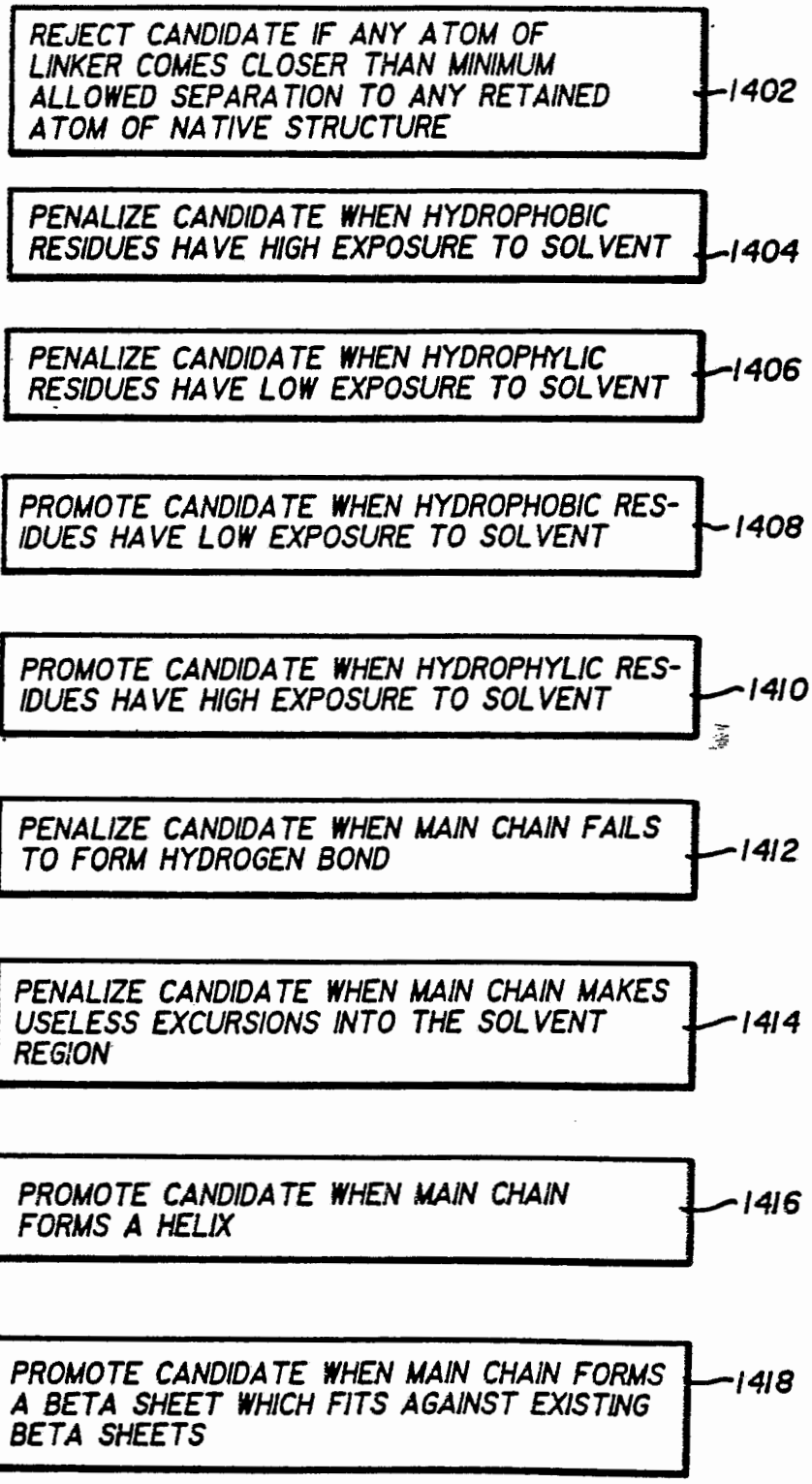

REJECT CANDIDATE IF ANY ATOM OF LINKER COMES CLOSER THAN MINIMUM ALLOWED SEPARATION TO ANY RETAINED ATOM OF NATIVE STRUCTURE
1402
PENALIZE CANDIDATE WHEN HYDROPHOBIC RESIDUES HAVE HIGH EXPOSURE TO SOLVENT
1404
PENALIZE CANDIDATE WHEN HYDROPHYLIC RESIDUES HAVE LOW EXPOSURE TO SOLVENT
1406
PROMOTE CANDIDATE WHEN HYDROPHOBIC RESIDUES HAVE LOW EXPOSURE TO SOLVENT
1408
PROMOTE CANDIDATE WHEN HYDROPHYLIC RESIDUES HAVE HIGH EXPOSURE TO SOLVENT
1410
PENALIZE CANDIDATE WHEN MAIN CHAIN FAILS TO FORM HYDROGEN BOND
1412
PENALIZE CANDIDATE WHEN MAIN CHAIN MAKES USELESS EXCURSIONS INTO THE SOLVENT REGION
1414
PROMOTE CANDIDATE WHEN MAIN CHAIN FORMS A HELIX
1416
PROMOTE CANDIDATE WHEN MAIN CHAIN FORMS A BETA SHEET WHICH FITS AGAINST EXISTING BETA SHEETS
1418

FIG. 14

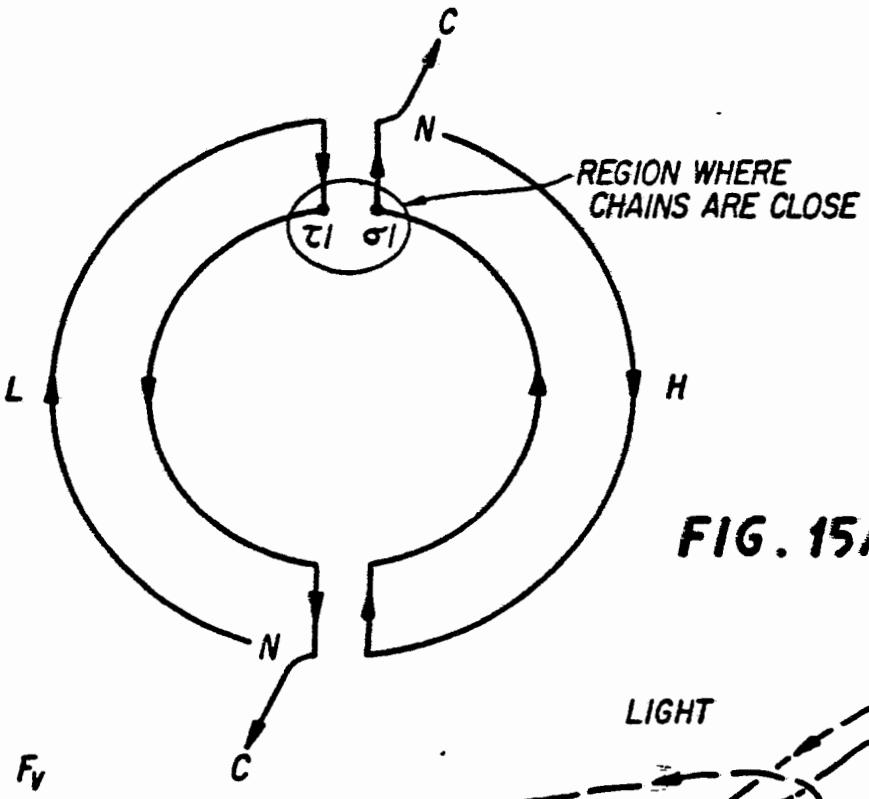


FIG. 15A

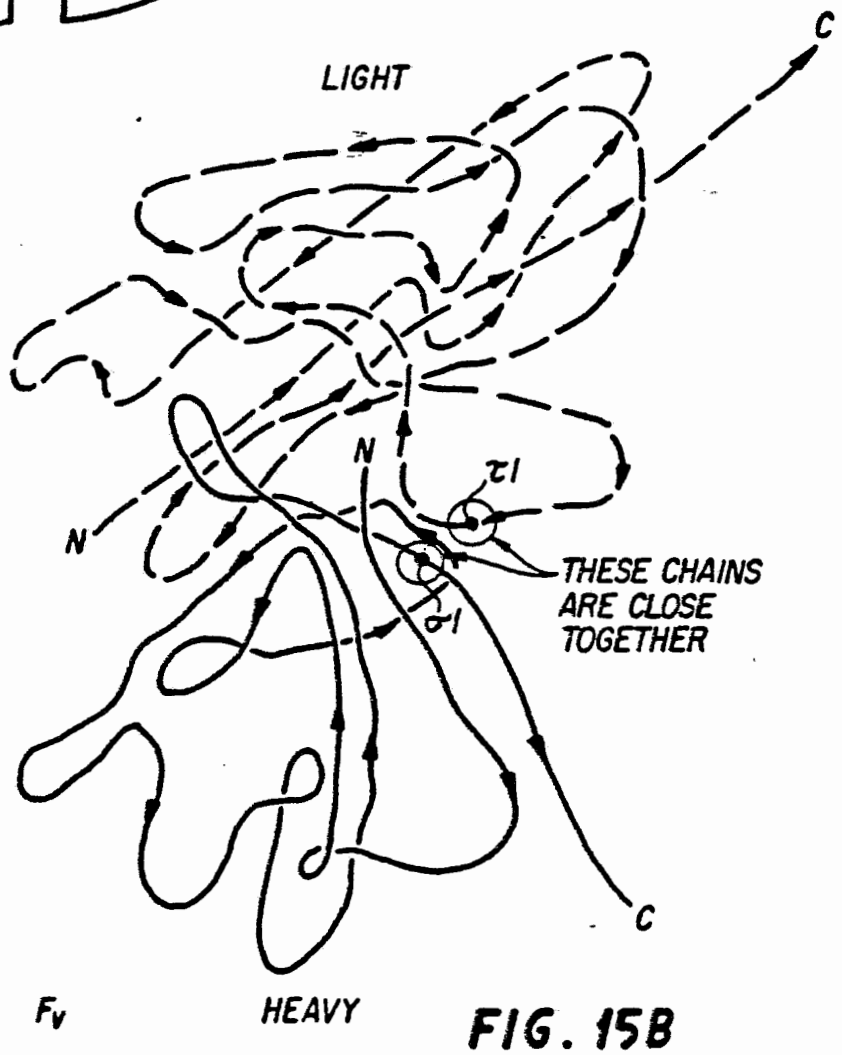


FIG. 15B

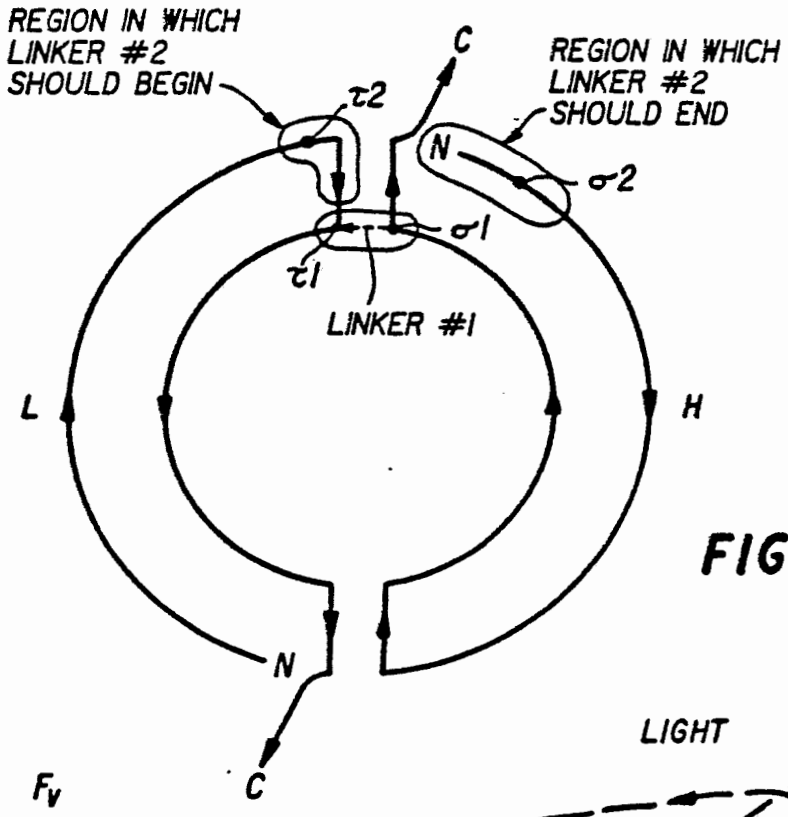


FIG. 16A

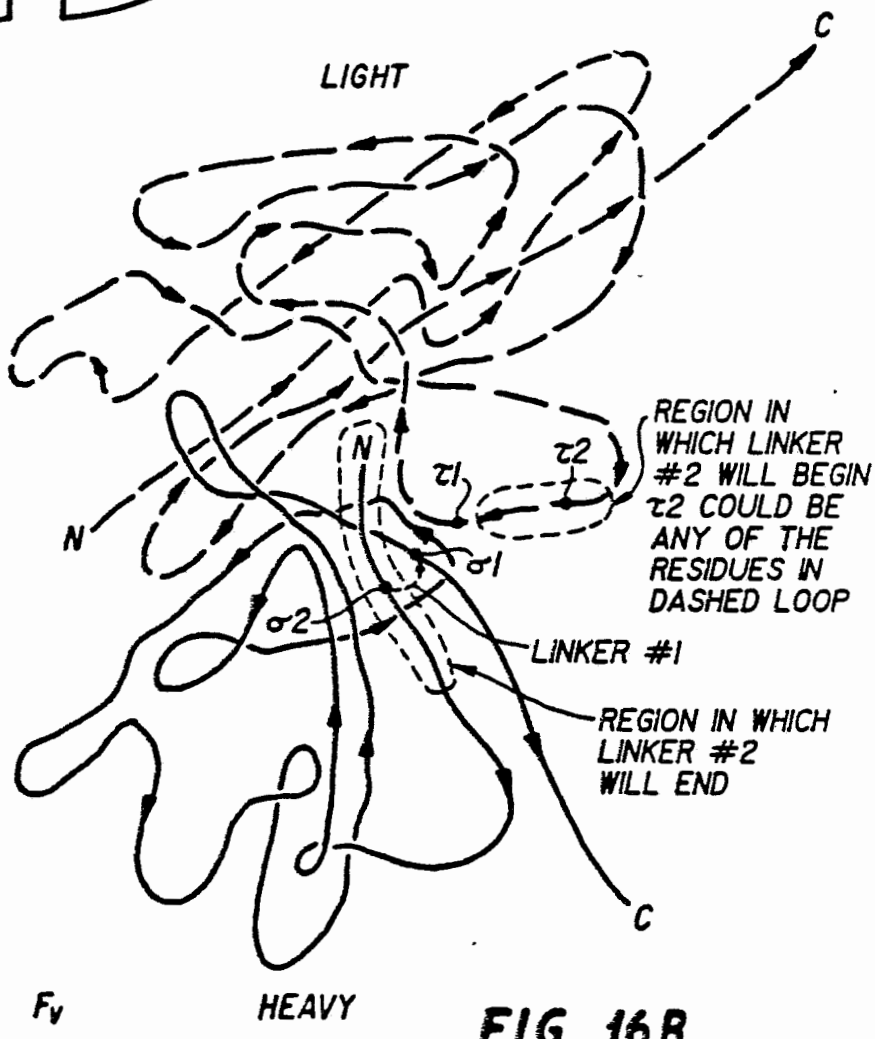


FIG. 16B

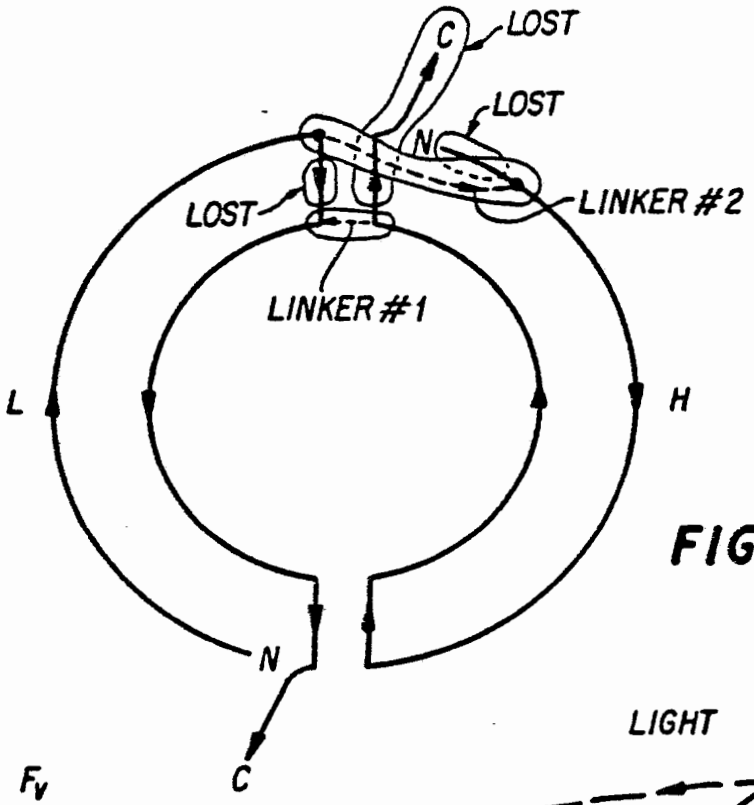


FIG. 17A

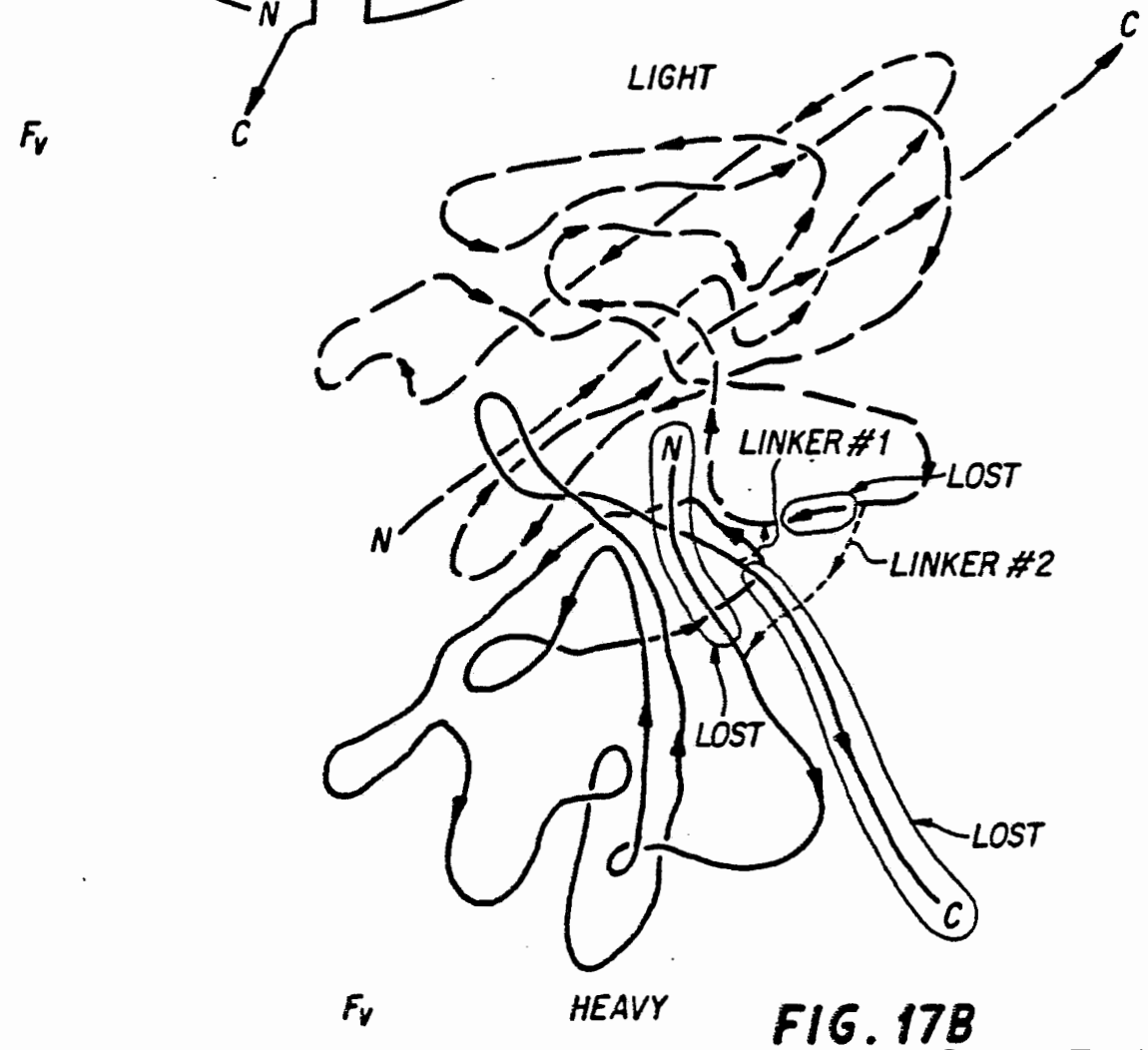


FIG. 17B

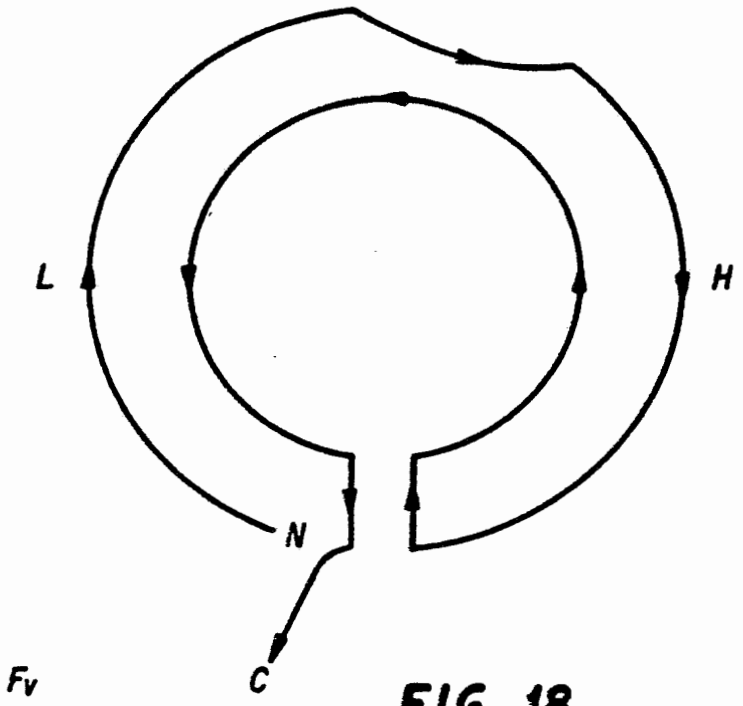


FIG. 18

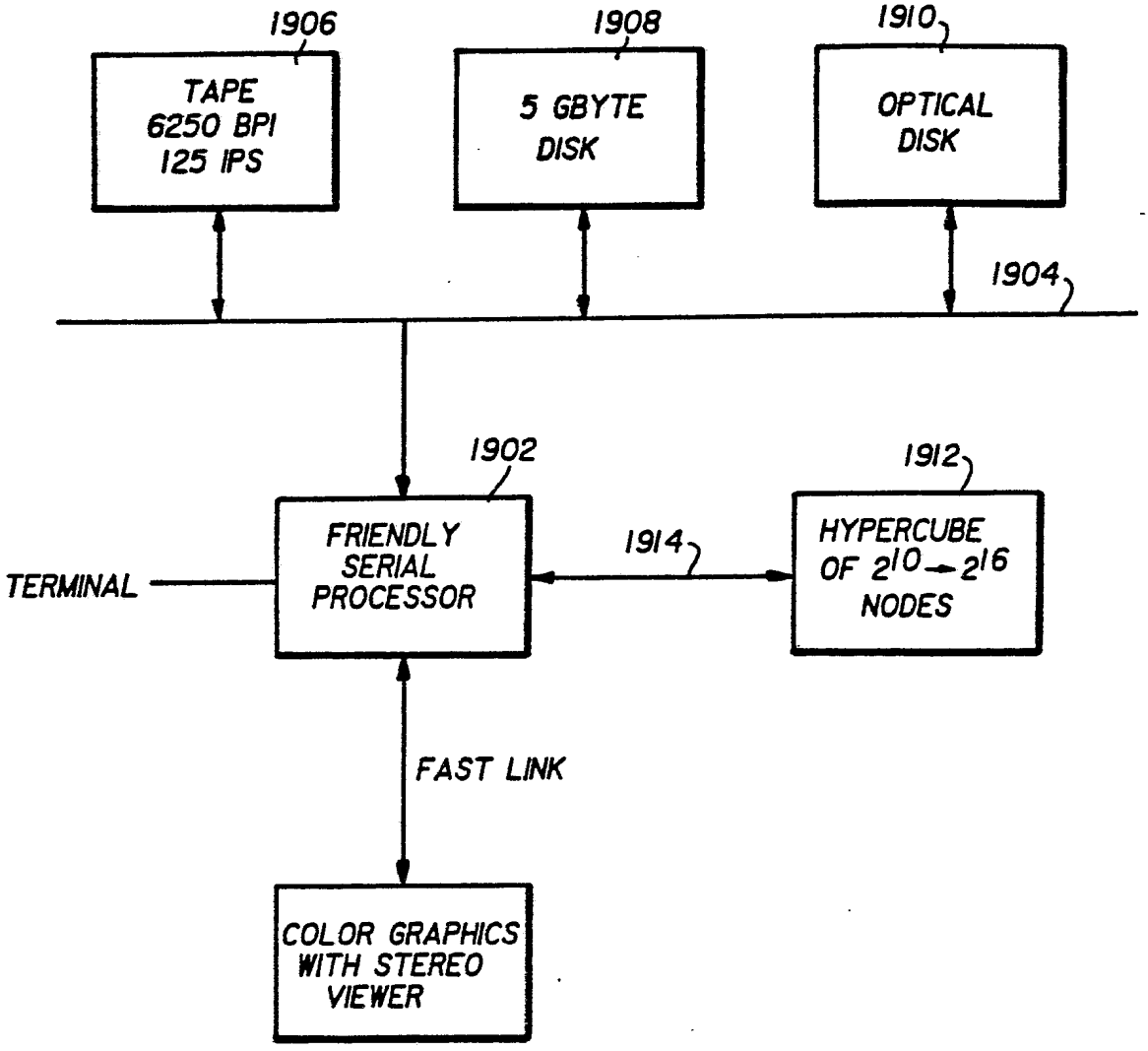


FIG. 19

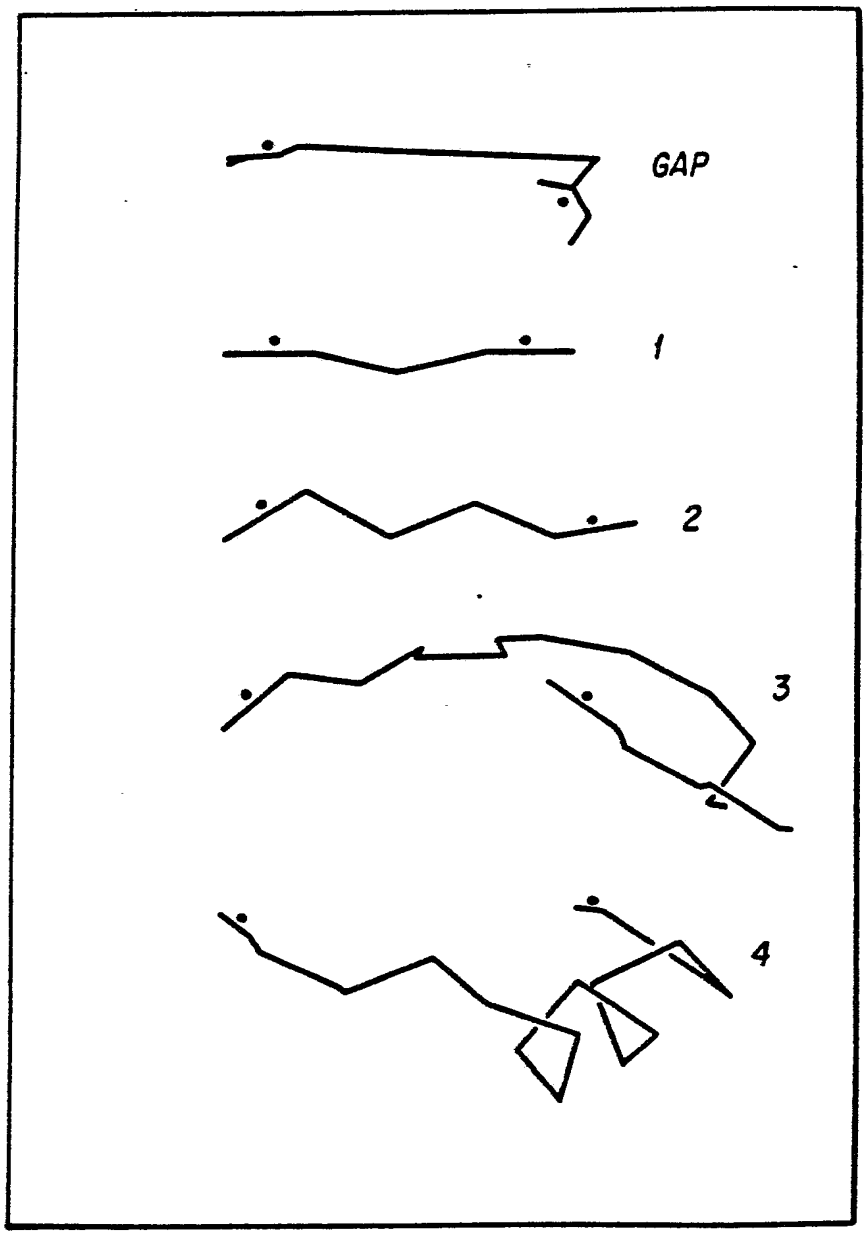


FIG. 20A

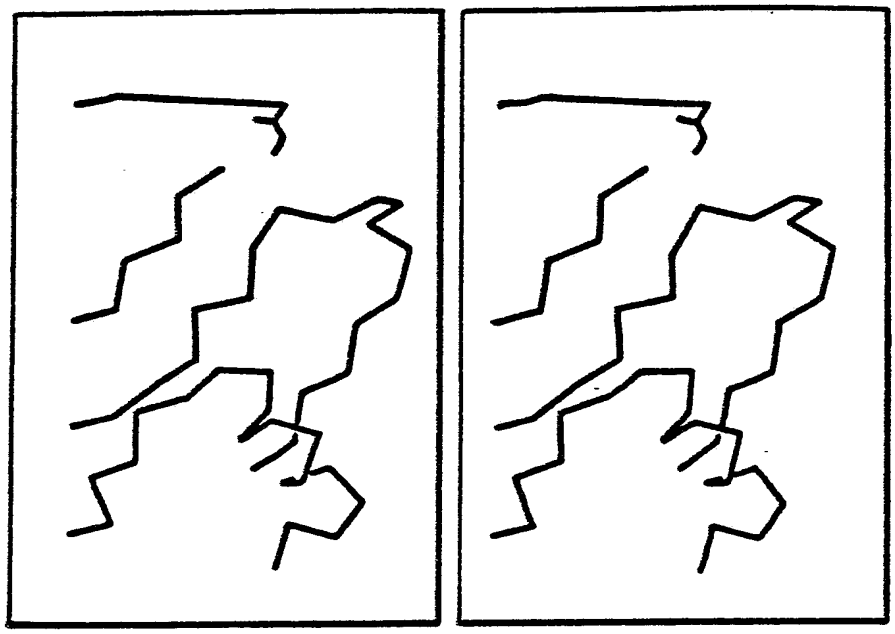


FIG. 20B

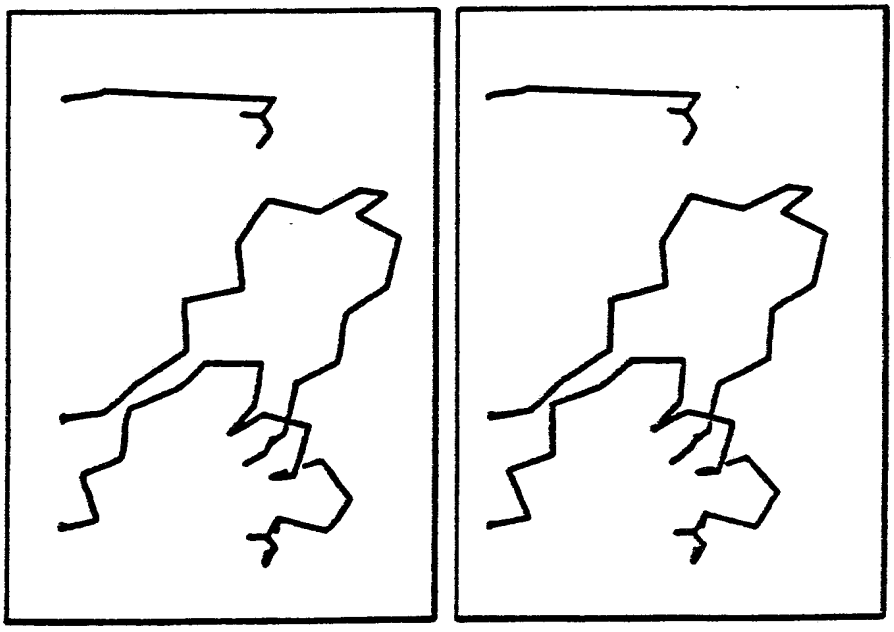


FIG. 20C

```
                    5                  10                  15                  20
Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys Pro Gly Gly Ser Leu Lys Leu
GAG GTG CAC CTG GTG GAG TCT GGG GGA GAC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC
                25                  30                  35                  40
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ile Ser Tyr Gly Met Ser Trp Val Arg Gln Thr
TCC TGT GCA GCC TCT GGA TTC ACT TTC ATT AGC TAT GGC ATG TCT TGG GTT CGC CAG ACT
            45                  50                  55                  60
Pro Asp Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly Ser Thr Tyr Thr Tyr Tyr
CCA GAC AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT AGT ACT TAC ACC TAC TAT
        65                  70                  75                  80
Pro Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
CCA GAC AGT GTG AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC
                85                  90                  95                  100
Leu Gln Met Ser Gly Leu Lys Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Ile
CTG CAA ATG AGC GGT CTG AAG TCT GAG GAC ACA GCC ATG TAT TAC TGT GCA AGA CGG ATT
            105                 110                 115                 120
Thr Thr Val Val Leu Thr Asp Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
AGT ACG GTA GTA CTT ACG GAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC
            125                 130                 135                 140
Thr Val Ser Ser Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro leu Ala Pro Gly Ser Ala
ACC GTC TCC TCA GCC AAA ACG ACA CCC CCA TCT GTC TAT CCA CTG GCC CCT GGA TCT GCT
        145                 150                 155                 160
Ala Gln Thr Asn Ser Met Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro
GCC CAA ACT AAC TCG ATG GTG ACC CTG GGA TGC CTG GTC AAG GGC TAT TTC CCT GAG CCA
                165                 170                 175                 180
Val Thr Val Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly Val His Thr Phe Pro Ala Val
GTG ACA GTG ACC TGG AAC TCT GGA TCC CTG TCC AGC GGT GTG CAC ACC TTC CCA GCT GTC
            185                 190                 195                 200
Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro
CTG CAG TCT GAC CTC TAC ACT CTG AGC AGC TCA GTG ACT GTG CCC TCC AGC ACC TGG CCC
            205                 210                 215                 220
Ser Glu Thr Val Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val Asp Lys Lys
AGC GAG ACC GTC ACC TGC AAC GTT GCC CAC CCG GCG AGC ACC AAG GTG GAC AAG AAA
        225                 230                 235                 240
Ile Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val Ser Ser
ATT GTG CCC AGG GAT TGT GGT TGT AAG CCT TGC ATA TGT ACA GTC CCA GAA GTA TCA TCT
                245                 250                 255                 260
Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile Thr Leu Thr Pro Lys Val
GTC TTC ATC TTC CCC CCA AAG CCC AAG GAT GTG CTC ACC ATT ACT CTG ACT CCT AAG GTC
            265                 270                 275                 280
Thr Cys Val Val Val Asp Ile Ser Lys Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val
ACG TGT GTT GTG GTA GAC ATC AGC AAG GAT GAT CCC GAG GTC CAG TTC AGC TGG TTT GTA
            285                 290                 295                 300
Asp Asp Val Glu Val His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr
GAT GAT GTG GAG CTG CAC ACA GCT CAG ACG CAA CCC CGG GAG GAG CAG TTC AAC AGC ACT
        305                 310                 315                 320
Ser Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys Glu Phe
TCC CGC TCA GTC AGT GAA CTT CCC ATC ATG CAC CAG GAC TGG CTC AAT GGC AAG GAG TTC
                325                 330                 335                 340
Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr
AAA TGC AGG GTC AAC AGT GCA GCT TTC CCT GCC CCC ATC GAG AAA ACC ATC TCC AAA ACC
            345                 350                 355                 360
Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala
AAA GGC AGA CCG AAG GCT CCA CAG GTG TAC ACC ATT CCA CCT CCC AAG GAG CAG ATG GCC
            365                 370                 375                 380
Lys Asp Lys Val Ser Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val
AAG GAT AAA GTC AGT CTG ACC TGC ATG ATA ACA GAC TTC TTC CCT GAA GAC ATT ACT GTG
        385                 390                 395                 400
Glu Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Arg Ile Met Asn
GAG TGG CAG TGG AAT GGG CAG CCA GCG GAG AAC TAC AAG AAC ACT CAG CGC ATC ATG AAC
                405                 410                 415                 420
Thr Asn Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln Lys Ser Asn Trp Glu Ala
ACG AAT GGC TCT TAC TTC GTC TAC AGC AAG CTC AAT GTG CAG AAG AGC AAC TGG GAG GCA
            425                 430                 435                 440
Gly Asn Thr Phe Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His Thr Glu Lys
GGA AAT ACT TTC ACC TGC TCT GTG TTA CAT GAG GGC CTG CAC AAC CAC CAT ACT GAG AAG
            445
Ser Leu Ser His Ser Pro Gly Lys ***
AGC CTC TCC CAC TCT CCT GGT AAA TGA
```

FIG. 21

				5					10					15					20
Glu	Asn	Val	Leu	Thr	Gln	Ser	Pro	Ala	Ile	Met	Ser	Ala	Ser	Pro	Gly	Glu	Lys	VAL	Thr
GAA	AAT	GTG	CTC	ACC	CAG	TCT	CCA	GCA	ATC	ATG	TCT	GCA	TCT	CCA	GGG	GAA	AAG	GTC	ACC
				25					30					35					40
Met	Thr	Cys	Arg	Ala	Ser	Ser	Ser	Val	Ser	Ser	Ser	Tyr	Leu	His	Trp	Phe	Gln	Gln	Lys
ATG	ACC	TGC	AGG	GCC	AGC	TCA	AGT	GTA	AGT	TCC	AGT	TAC	TTG	CAC	TGG	TTC	CAG	CAG	AAG
				45					50					55					60
Ser	Gly	Ala	Ser	Pro	Lys	Leu	Trp	Ile	Tyr	Ser	Thr	Ser	Asn	Leu	Ala	Ser	Gly	Val	Pro
TCA	GGT	GCC	TCC	CCC	AAA	CTC	TGG	ATT	TAT	AGC	ACA	TCC	AAC	TTG	GCT	TCT	GGA	GTC	CCT
				65					70					75					80
Ala	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Ser	Tyr	Ser	Leu	Thr	Ile	Ser	Ser	Val	Glu
GCT	CGC	TTC	AGT	GGC	AGT	GGG	TCT	GGG	ACC	TCT	TAC	TCT	CTC	ACA	ATC	AGC	AGT	GTG	GAG
				85					90					95					100
Ala	Glu	Asp	Ala	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Tyr	Ser	Gly	Tyr	Pro	Leu	Thr	Phe	Gly
GCT	GAA	GAT	GCT	GCC	ACT	TAT	TAC	TGC	CAG	CAG	TAC	AGT	GGT	TAC	CCA	CTC	ACG	TTC	GGT
				105					110					115					120
Ala	Gly	Thr	Lys	Leu	Glu	Leu	Lys	Arg	Ala	Asp	Ala	Ala	Pro	Thr	Val	Ser	Ile	Phe	Pro
GCT	GGG	ACC	AAG	CTG	GAG	CTG	AAA	CGG	GCT	GAT	GCT	GCA	CCA	ACT	GTA	TCC	ATC	TTC	CCA
				125					130					135					140
Pro	Ser	Ser	Glu	Gln	Leu	Thr	Ser	Gly	Gly	Ala	Ser	Val	Val	Cys	Phe	Leu	Asn	Asn	Phe
CCA	TCC	AGT	GAG	CAG	TTA	ACA	TCT	GGA	GGT	GCC	TCA	GTC	GTG	TGC	TTC	TTG	AAC	AAC	TTC
				145					150					155					160
Tyr	Pro	Lys	Asp	Ile	Asn	Val	Lys	Trp	Lys	Ile	Asp	Gly	Ser	Glu	Arg	Gln	Asp	Gly	Val
TAC	CCC	AAA	GAC	ATC	AAT	GTC	AAG	TGG	AAG	ATT	GAT	GGC	AGT	GAA	CGA	CAA	AAT	GGC	GTC
				165					170					175					180
Leu	Asn	Ser	Trp	Thr	Asp	Gln	Asp	Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Met	Ser	Ser	Thr	Leu
CTG	AAC	AGT	TGG	ACT	GAT	CAG	GAC	AGC	AAA	GAC	AGC	ACC	TAC	AGC	ATG	AGC	AGC	ACC	CTC
				185					190					195					200
Thr	Leu	Thr	Lys	Asp	Glu	Tyr	Glu	Arg	His	Asn	Ser	Tyr	Thr	Cys	Glu	Ala	Thr	His	Lys
ATG	TTG	ACC	AAG	GAC	GAG	TAT	GAA	CGA	CAT	AAC	AGC	TAT	ACC	TGT	GAG	GCC	ACT	CAC	AAG
				205					210					215					
Thr	Ser	Thr	Ser	Pro	Ile	Val	Lys	Ser	Phe	Asn	Arg	Asn	Glu	Cys	***				
ACA	TCA	ACT	TCA	CCC	ATT	GTC	AAG	AGC	TTC	AAC	AGG	AAT	GAG	TGT	TAG				

FIG. 22

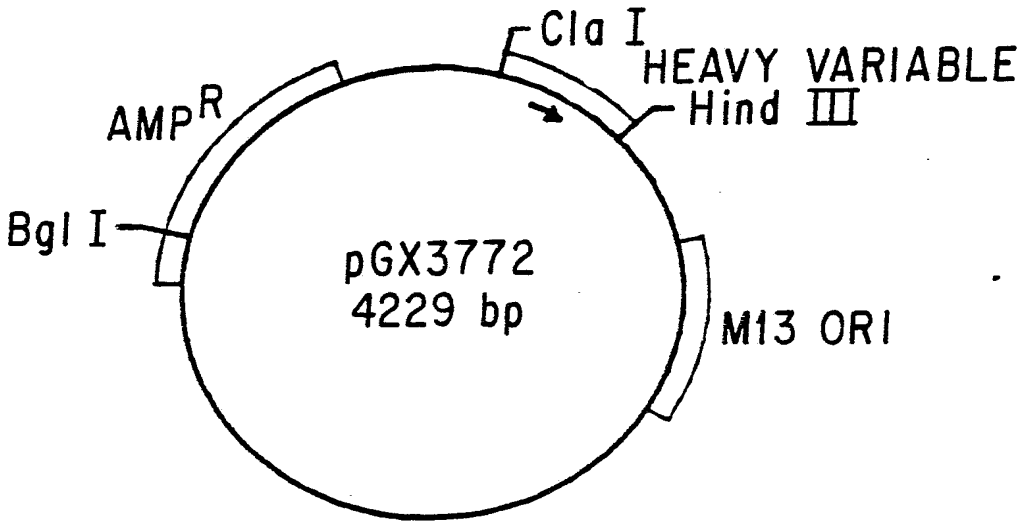


FIG. 23A

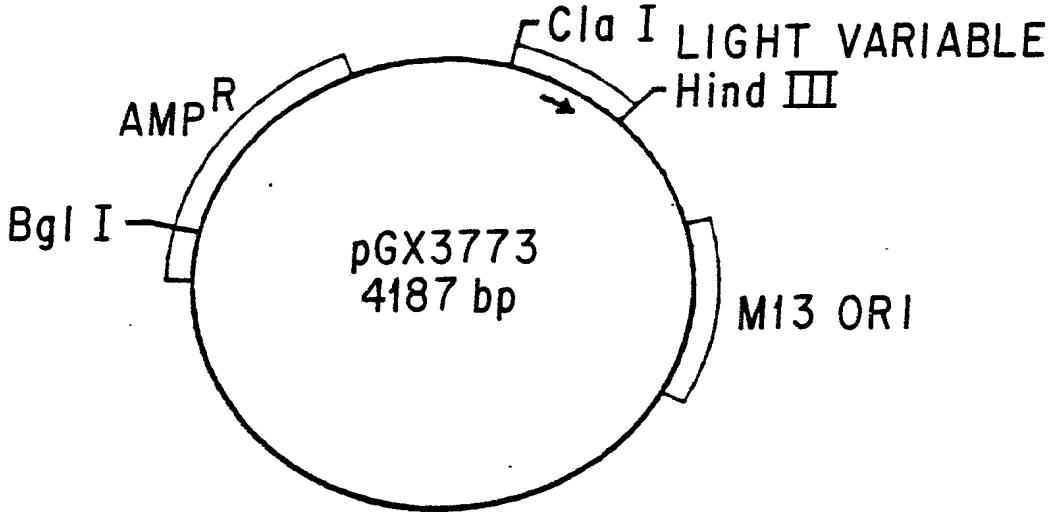


FIG. 23B

```
                    5                   10                  15                  20
Met Glu Asn Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys Val
ATG GAA AAT GTG CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAA AAG GTC

                25                  30                  35                  40
Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Ser Tyr Leu His Trp Phe Gln Gln
ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC AGT TAC TTG CAC TGG TTC CAG CAG

            45                  50                  55                  60
Lys Ser Ile Ala Lys Ala Phe Lys Asn Gly Gly Asp Leu Val Lys Pro Gly Gly Ser Leu
AAG TCA ATC GCG AAA GCG TTC AAA AAC GGG GGA GAC TTA GTG AAG CCT GGA GGG TCC CTG

        65                  70                  75                  80
Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ile Ser Tyr Gly Met Ser Trp Val Arg
AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC ATT AGC TAT GGC ATG TCT TGG GTT CGC

                85                  90                  95                  100
Gln Thr Pro Asp Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly Ser Thr Tyr Thr
CAG ACT CCA GAC AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT GGT GGT AGT ACT TAC ACC

            105                 110                 115                 120
Tyr Tyr Pro Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr
TAC TAT CCA GAC AGT GTG AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC

        125                 130                 135                 140
Leu Tyr Leu Gln Met Ser Gly Leu Lys Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg
CTG TAC CTG CAA ATG AGC GGT CTG AAG TCT GAG GAC ACA GCC ATG TAT TAC TGT GCA AGA

            145                 150                 155                 160
Arg Ile Thr Thr Val Val Leu Thr Asp Tyr Tyr Ala Met Asp Tyr Trp Gly Pro Gly Ser
CGG ATT ACT ACG GTA GTA CTT ACG GAT TAC TAT GCT ATG GAC TAC TGG GGT CCG GGT TCT

            165                 170                 175                 180
Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
CCC AAA CTC TGG ATT TAT AGC ACA TCC AAC TTG GCT TCT GGA GTC CCT GCT CGC TTC AGT

            185                 190                 195                 200
Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val Glu Ala Glu Asp Ala
GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG GAG GCT GAA GAT GCT

            205                 210                 215                 220
Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys
GCC ACT TAT TAC TGC CAG CAG TAC AGT GGT TAC CCA CTC ACG TTC GGT GCT GGG ACC AAG

        225
Leu Glu Leu Lys Arg ***
CTG GAG CTG AAA CGG TAA
```

FIG. 24

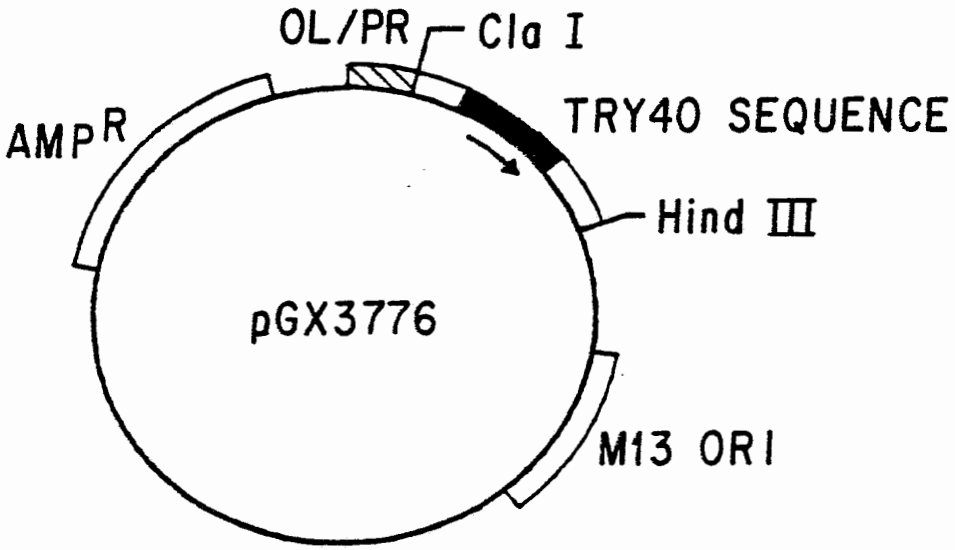

OL/PR
Cla I
AMPR
TRY40 SEQUENCE
Hind III
pGX3776
M13 ORI

FIG. 25

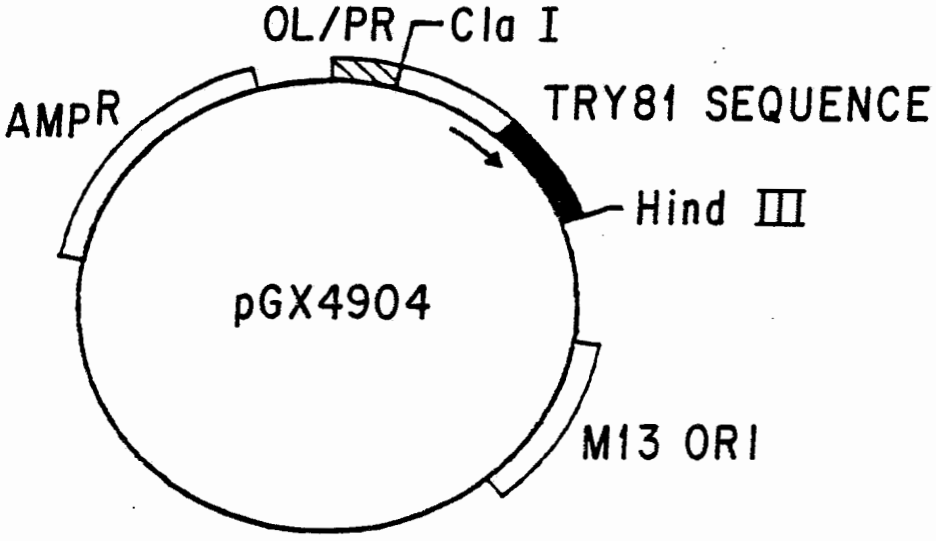

OL/PR
Cla I
AMPR
TRY81 SEQUENCE
Hind III
pGX4904
M13 ORI

FIG. 27

TRY61
sca try 61

```
                    5                  10                  15                  20
Met Glu Asn Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys Val
ATG GAA AAT GTG CTC ACC CAG TCT CGA GCA ATC ATG TCT GCA TCT CCA GGG GAA AAG GTC

                   25                  30                  35                  40
Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Ser Tyr Leu His Trp Phe Gln Gln
ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC AGT TAC TTG CAC TGG TTC CAG CAG

                   45                  50                  55                  60
Lys Ser Gly Ala Ser Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val
AAG TCA GGT GCC TCC CCC AAA CTC TGG ATT TAT AGC ACA TCC AAC TTC GCT TCT GGA GTC

                   65                  70                  75                  80
Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val
CCT GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG

                   85                  90                  95                 100
Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro Leu Thr Phe
GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG TAC AGT GGT TGT TAC CCA CTC ACG TTC

                  105                 110                 115                 120
Gly Ala Gly Thr Lys Val Arg Gly Ser Pro Ala Ile Asn Val Ala Val His Val Phe Ser
GGT GCT GGG ACC AAG GTT CGT GGT TCT CCG GCA ATC AAC GTA GCT GTA CAC GTA TTC TCT

                  125                 130                 135                 140
Gly Gly Asp Leu Val Lys Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe
GGG GGA GAC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC

                  145                 150                 155                 160
Thr Phe Ile Ser Tyr Gly Met Ser Trp Val Arg Gln Thr Pro Asp Lys Arg Leu Glu Trp
ACT TTC ATT AGC TAT GGC ATG TCT TGG GTT CGC CAG ACT CCA GAC AAG AGG CTG GAG TGG

                  165                 170                 175                 180
Val Ala Thr Ile Ser Ser Gly Ser Thr Tyr Thr Tyr Tyr Pro Asp Ser Val Lys Gly Arg
GTC GCA ACC ATT AGT AGT GGT AGT ACT TAC ACC TAC TAT CCA GAC AGT GTG AAG GGG CGA

                  185                 190                 195                 200
Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr Leu Gln Met Ser Gly Leu Lys
TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC CTG CAA ATG AGC GGT CTG AAG

                  205                 210                 215                 220
Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Ile Thr Thr Val Val Leu Thr Asp
TCT GAG GAC ACA GCC ATG TAT TAC TGT GCA AGA CGG ATT ACT ACG GTA GTA CTT ACG GAT

                  225                 230                 235
Tyr Tyr Ala Met Asp Tyr Trp Gly Thr Gly Thr Ser Val Thr Val Ser ***
TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TAA
```

FIG. 26

```
                    5                  10                  15                  20
Met Glu Asn Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys Val
ATG GAA AAT GTG CTC ACC CAG TCT CGA GCA ATC ATG TCT GCA TCT CCA GGG GAA AAG GTC

                   25                  30                  35                  40
Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Ser Tyr Leu His Trp Phe Gln Gln
ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC AGT TAC TTG CAC TGG TTC CAG CAG

                   45                  50                  55                  60
Lys Ser Gly Ala Ser Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val
AAG TCA GGT GCC TCC CCC AAA CTC TGG ATT TAT AGC ACA TCC AAC TTC GCT TCT GGA GTC

                   65                  70                  75                  80
Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val
CCT GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG

                   85                  90                  95                  100
Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro Leu Thr Phe
GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG TAC AGT GGT GGT TAC CCA CTC ACG TTC

                  105                 110                 115                 120
Gly Ala Gly Thr Lys Leu Lys Glu Ser Gly Ser Val Ser Ser Glu Gln Leu Ala Gln Phe
GGT GCT GGG ACC AAG CTG AAA GAA TCT GGT TCT GTT TCT TCT GAA CAG CTG GCT CAG TTT

                  125                 130                 135                 140
Arg Ser Leu Asp Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys Pro Gly Gly Ser
CGT TCT CTG GAT GTG CAG CTG GTG GAG TCT GGG GGA GAC TTA GTG AAG CCT GGA GGG TCC

                  145                 150                 155                 160
Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ile Ser Tyr Gly Met Ser Trp Val
CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC ATT AGC TAT GGC ATG TCT TGG GTT

                  165                 170                 175                 180
Arg Gln Thr Pro Asp Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly Ser Thr Tyr
CGC CGA ACT CCA GAC AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT AGT ACT TAC

                  185                 190                 195                 200
Thr Tyr Tyr Pro Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
ACC TAC TAT CCA GAC AGT GTG AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC

                  205                 210                 215                 220
Thr Leu Tyr Leu Gln Met Ser Gly Leu Lys Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala
ACC CTG TAC CTG CAA ATG AGC GGT CTG AAG TCT GAG GAC ACA GCC ATG TAT TAC TGT GCA

                  225                 230                 235                 240
Arg Arg Ile Thr Thr Val Val Leu Thr Asp Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly
AGA CGG ATT ACT ACG GTA GTA CTT ACG GAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA

                  245
Thr Ser Val Thr Val Ser ***
ACC TCA GTC ACC GTC TCC TAA
```

FIG. 28

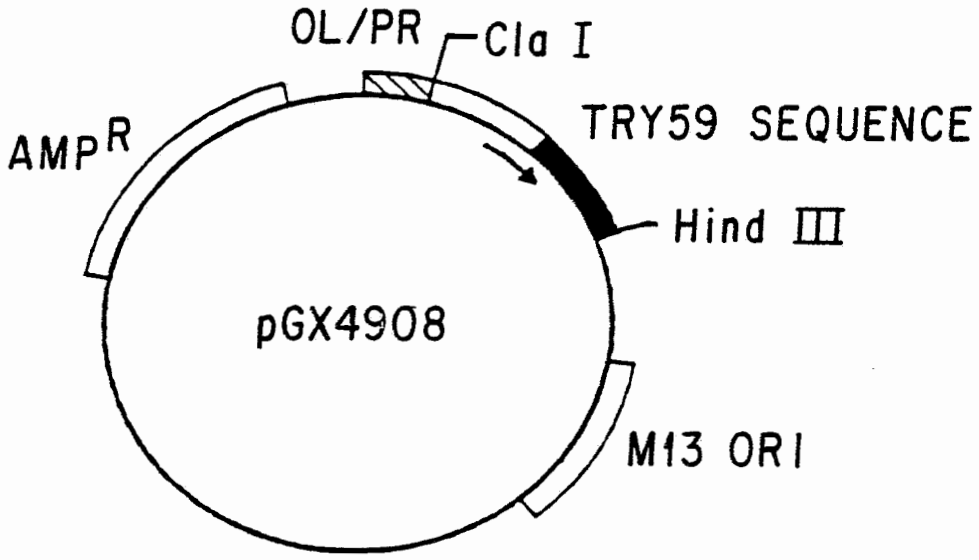


FIG. 29

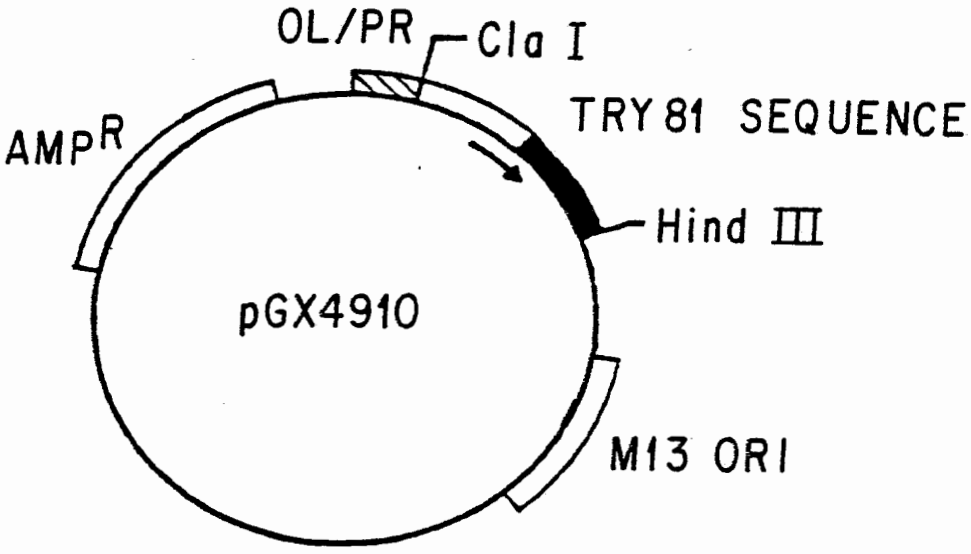


FIG. 34

FIG. 30A

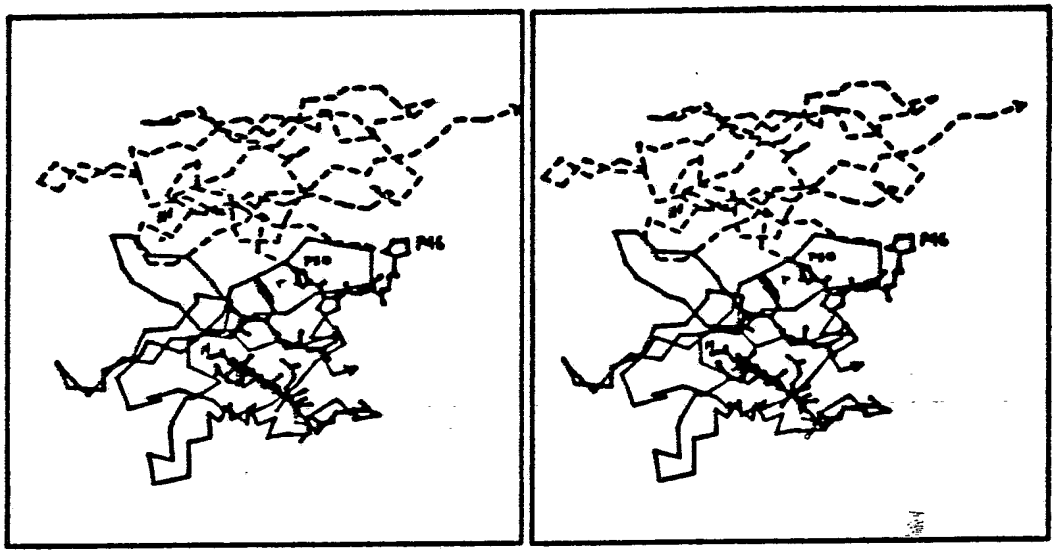


FIG. 30B

FIG. 30C

FIG. 30D

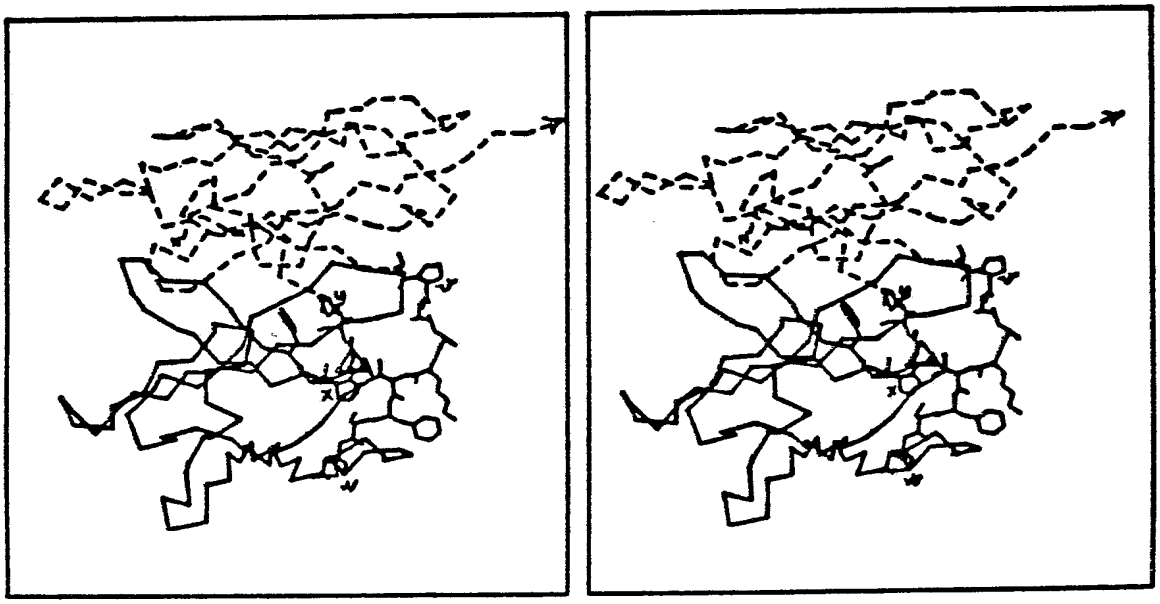


FIG. 31A

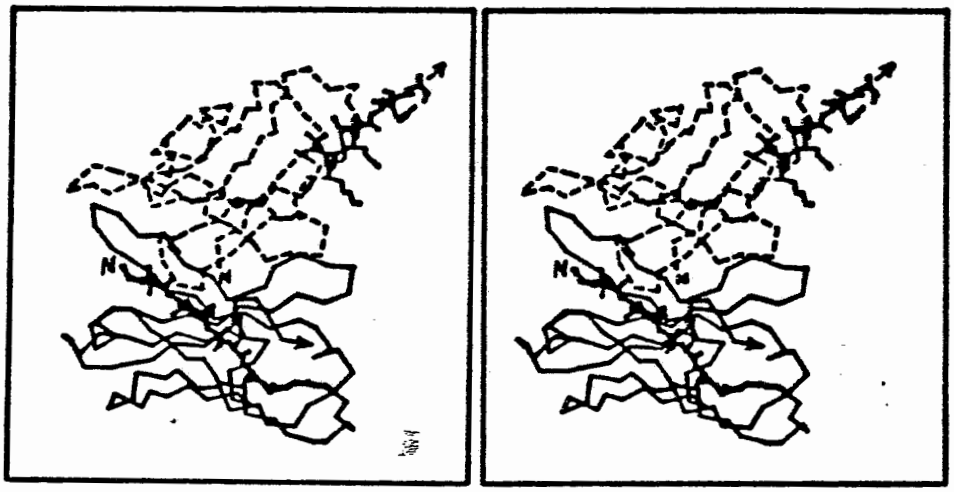


FIG. 31B

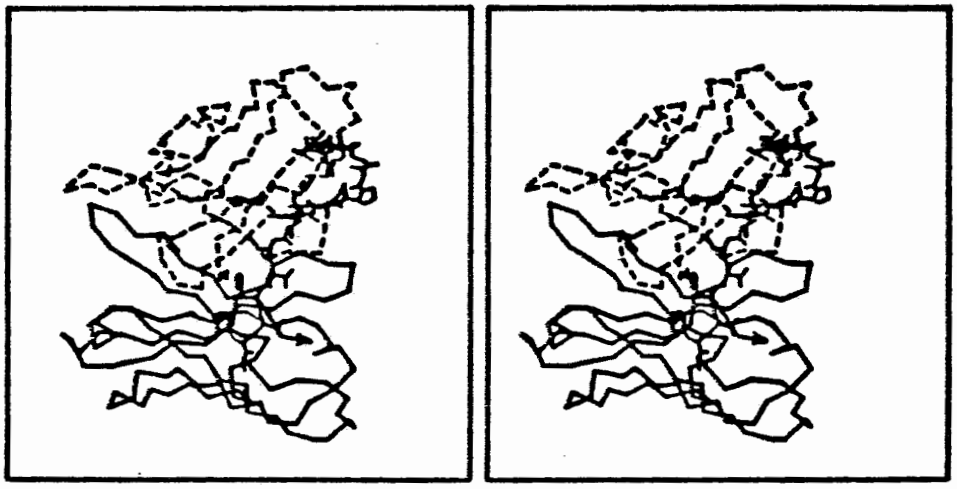


FIG. 32A

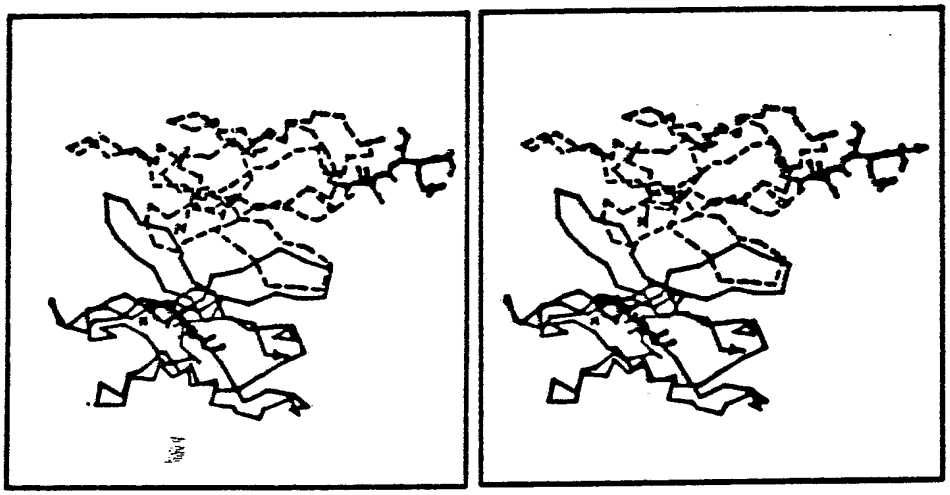
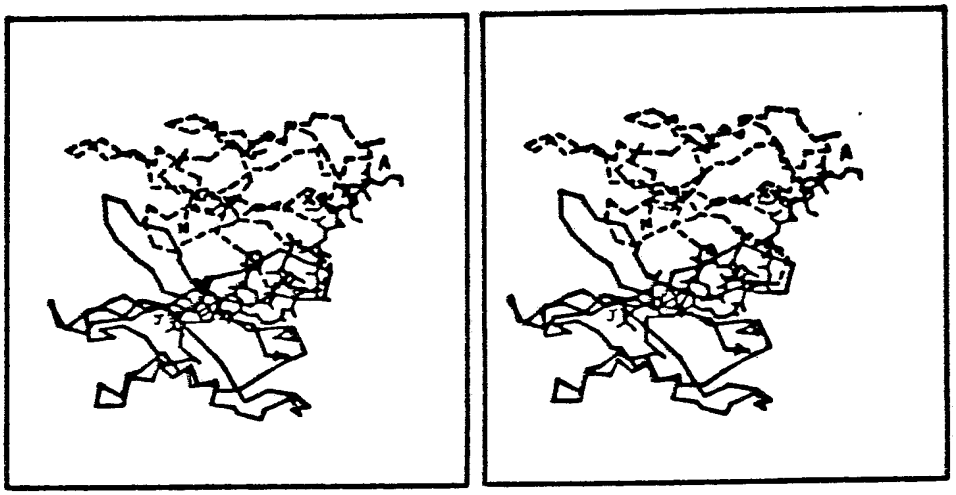


FIG. 32B



TRY048
SCA TRY1048, ALL OF VL AND VH

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                    5                  10                  15                  20
Met Glu Asn Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys Val
ATG GAA AAT GTG CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAA AAG GTC
                   25                  30                  35                  40
Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Ser Tyr Leu His Trp Phe Gln Gln
ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC AGT TCC AGT TAC TGG TTC CAG CAG
                   45                  50                  55                  60
Lys Ser Gly Ala Ser Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val
AAG TCA GGT GCC TCC CCC AAA CTC TGG ATT TAT AGC ACA TCC AAC TTG GCT TCT GGA GTC
                   65                  70                  75                  80
Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val
CCT GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG
                   85                  90                  95                 100
Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro Leu Thr Phe
GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TAC AGT GGT TAC CCA CTC ACG TTC
                  105                 110                 115                 120
Gly Ala Gly Thr Lys Leu Glu Ala Glu Gly Thr Leu Ser Pro Ala Asp Lys Thr Asn Val
GGT GCT GGG ACC AAG CTG GAG GCA GAA GGC ACT CTG TCT CCA GCA GAT AAA ACT AAC GTT
                  125                 130                 135                 140
Lys Ala Ala Trp Gly Lys Val Met Thr Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys
AAA GCA GCA TGG GGC AAA GTT ATG ACT CAG CTG GTG GAG TCT GGG GGA GAC TTA GTG AAG
                  145                 150                 155                 160
Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ile Ser Tyr Gly
CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA GCA TCT GGA TTC ACT TTC ATT AGC TAT GGC
                  165                 170                 175                 180
Met Ser Trp Val Arg Gln Thr Pro Asp Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser
ATG TCT TGG GTT CGC CAG ACT CCA GAC AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT
                  185                 190                 195                 200
Gly Ser Thr Tyr Thr Tyr Tyr Pro Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp
GGT AGT ACT TAC ACC TAC TAT CCA GAC AGT GTG AAG GGG CGA TTC ACC ATC TCC AGA GAC
                  205                 210                 215                 220
Asn Ala Lys Asn Thr Leu Tyr Leu Gln Met Ser Gly Leu Lys Ser Glu Asp Thr Ala Met
AAT GCC AAG AAC ACC CTG TAC CTG CAA ATG AGC GGT CTG AAG TCT GAG GAC ACA GCC ATG
                  225                 230                 235                 240
Tyr Tyr Cys Ala Arg Arg Ile Thr Thr Val Val Leu Thr Asp Tyr Tyr Ala Met Asp Tyr
TAT TAC TGT GCA AGA CGG ATT ACT ACG GTA GTA CTT ACG GAT TAC TAT GCT ATG GAC TAC
                  245                 250
Trp Gly Gln Gly Thr Ser Val Thr Val Ser ***
TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TAA
```

FIG. 33

FIG. 35

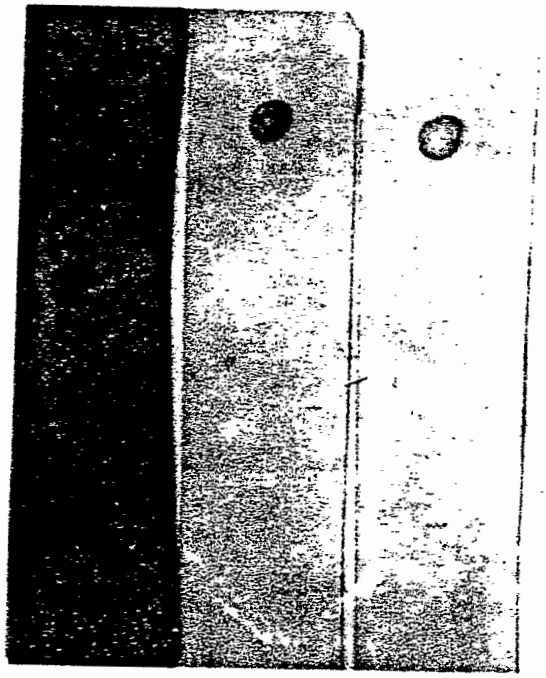
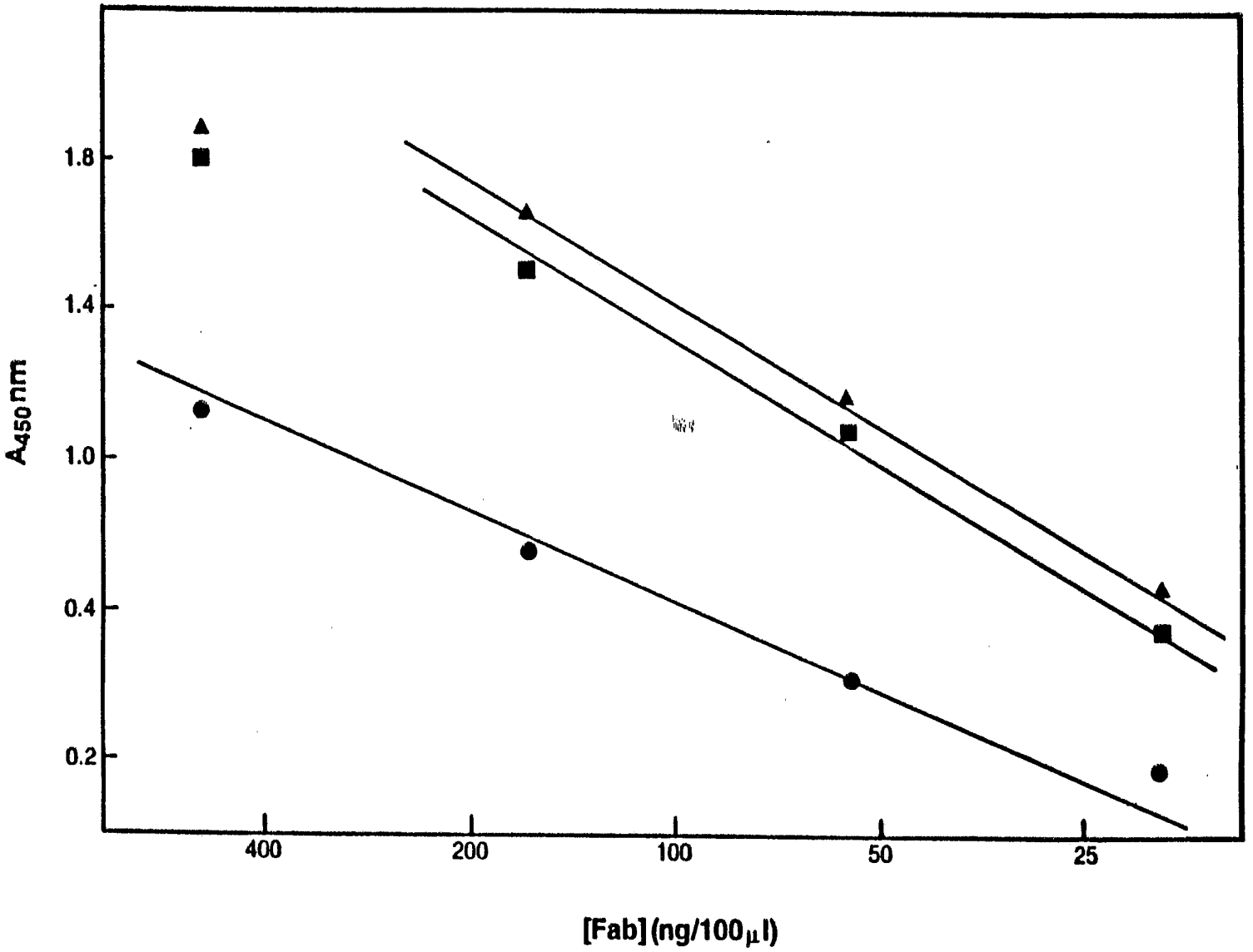


FIG. 36

Competitive Elisa



INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/02208

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.Cl.: 435/68 536/27 530/387 424/85

IPC(4): C12P 21/00 C07H 17/00 C07K 15/04 A61K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	435/7,68,91,172.3,240.2,253,254,255,320 530/387,388,389,390,391,413 536/27 424/85

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

Chemical Abstracts Data Base 1967-1987 Keywords: Antibod?, Variable Region, Conformation, FOLD?, Peptide, Linker, Heavy chain, Light chain, chimeric antibod?, monospecific antibod?

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
Y	GB, A, 2,137,631A (BOSS ET AL) 10 October 1984. See pages 3-6, 9-16 and claims 1-7 and 19-33.	1-27
Y	EP, A, 0,125,023 (CABILLY ET AL) 14 November 1984. See pages 3, 4, 15-18, and 21-29.	1-27
P, A	SCIENCE, Volume 81, issued August 1987, (Washington, D.C., USA) (R. HUBER) "Structural Basis for Antigen-Antibody Recognition", see pages 702-703.	1-11 and 21
A	BIOTECHNOLOGY, Volume 4, issued April 1986, (New York, New York, U.S.A.) (J. VAN BRUNT) "Protein Architecture: Designing From the Ground Up", See pages 277-283.	1-11 and 21

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
19 NOVEMBER 1987	10 DEC 1987
International Searching Authority 1	**Signature of Authorized Officer 20**
ISA/US	Jayme A. Huleatt

Form PCT/ISA/210 (second sheet) (May 1986)

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING (ATTACHMENT)

Group I, claims 1-11 and 21

Group II, claims 12-20

Group III, claim 26

Group IV, claim 27

A single inventive concept was not presented by these claims for the reasons outlined in Form PCT/ISA/206 mailed Oct. 22, 1987.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: David W. Staple
2275 Deming Way # 310
Middleton, Wisconsin 53562
United States of America

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 30 AUG 2016
Applicant's or agent's file reference UCHI34458WO	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US16/35228	International filing date *(day/month/year)* 01 June 2016 (01.06.2016)
Applicant THE UNIVERSITY OF CHICAGO	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

   Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

   Within **19 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference UCHI34458WO	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US16/35228	International filing date *(day/month/year)* 01 June 2016 (01.06.2016)	(Earliest) Priority Date *(day/month/year)* 01 June 2015 (01.06.2015)
Applicant THE UNIVERSITY OF CHICAGO		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of:
      ☒ the international application in the language in which it was filed.
      ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,
   a. the figure of the **drawings** to be published with the abstract is Figure No. 1
      ☒ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/35228

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 30-32, 35-56, 95-110, 113-150
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/35228

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 16/28, 14/00, 16/00; C07H 21/02; C12N 1/20; C12Q 1/68; A61K 39/39, 35/74 (2016.01)
CPC - C07K 16/00, 16/28; C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) Classifications: C07K 16/28, 14/00, 16/00; C07H 21/02; C12N 1/20; C12Q 1/68; A61K 39/39, 35/74; A01N 63/00 (2016.01)
CPC Classifications: C07K 16/00, 16/28; C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google Scholar, EBSCO, PubMed; Bifidobacterium, fecal, transplant, antibiotic, cancer, therapy, commensal, donor, checkpoint, inhibitor, RNA, formulate, probiotic, microbe, microbial, bacteria, microflora, microorganism, gut, stomach, intestine, gastrointestinal, digestive, immune, antibody, reaction, response, method

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 876 167 A1 (INSTITUT GUSTAVE ROUSSY) 27 May 2015; paragraphs [0046], [0053]-[0059], [0061], [0110], [0187]-[0188]	1-7, 9, 13-23, 26, 33-34, 57, 61-62, 64-65, 68-73, 75, 79-81, 83-85, 88-91
Y		--- 8, 10-12, 24-25, 27-28, 29/26-28, 58-60, 63, 74, 76-78, 66-67, 82, 86-87, 92-94, 111-112, 113/111-74
Y	(ZITVOGEL, L et al.) Cancer and the gut microbiota: An unexpected link. Science Translation Medicine. 21 January 2015. Vol 7. Issue 271; page 3, column 2, paragraph 2, lines 7-9	10-12
Y	WO 2014/145958 A4 (SERES HEALTH, INC.) 18 September 2014; paragraph [0187]	66-67, 82, 87, 111-112, 113/111-112
Y	WO 2011/068810 (SHIRE HUMAN GENETIC THERAPIES) 09 June 2011; page 8, line 32 to page 9, line 1	92-93
Y	US 7,195,906 B2 (COLLINS, JK et al.) 27 March 2007; column 4, lines 1-5; column 4, lines 64-65	8, 24-25, 27-28, 29/26-28, 58-60, 63, 76-78, 82
Y	WO 2015/061372 A1 (HEMOSHEAR, LLC) 30 April 2015; claim 174	74, 86, 94

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 2016 (09.08.2016)	30 AUG 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: David W. Staple
2275 Deming Way # 310
Middleton, Wisconsin 53562
United States of America

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* 30 AUG 2016

Applicant's or agent's file reference
UCHI34458WO

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US16/35228	01 June 2016 (01.06.2016)	01 June 2015 (01.06.2015)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C07K 16/28, 14/00, 16/00; C07H 21/02; C12N 1/20; C12Q 1/68; A61K 39/39, 35/74 (2016.01)
CPC - C07K 16/00, 16/28; C12Q 1/68

Applicant THE UNIVERSITY OF CHICAGO

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	9 August 2016 (09.08.2016)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 30-32, 35-56, 95-110, 113-150

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 30-32, 35-56, 95-110, 113-150 are so unclear that no meaningful opinion could be formed *(specify)*:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 30-32, 35-56, 95-110, 113-150

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	-***-Please See Below-***-	YES
	Claims	-***-Please See Below-***-	NO
Inventive step (IS)	Claims	NONE -	YES
	Claims	-***-Please See Below-***-	NO
Industrial applicability (IA)	Claims	-***-Please See Below-***-	YES
	Claims	NONE	NO

2. Citations and explanations:

-Continued from Box No. V: 1.:

Novelty (N): YES: Claims 8, 10-12, 24-25, 27-28, 29/26-28, 58-60, 63, 66-67, 74, 76-78, 82, 86-87, 92-94, 111-112, 113/111-74

Novelty (N): NO: Claims 1-7, 9, 13-23, 26, 33-34, 57, 61-62, 64-65, 68-73, 75, 79-81, 83-85, 88-91

Inventive step (IS): NO: Claims 1-28, 29/26-28, 33-34, 57-90, 91/89-90, 92-94, 111-112, 113/111-112

Industrial applicability (IA): YES: Claims 1-28, 29/26-28, 33-34, 57-90, 91/89-90, 92-94, 111-112, 113/111-112

Claims 1-7, 9, 13-23, 26, 33-34, 57, 61-62, 64-65, 68-73, 75, 79-81, 83-85, and 88-91 lack novelty under PCT Article 33(2) as being anticipated by EP 2 876 167 A1 to Institut Gustave Roussy (hereinafter "Zitvogel").

As per claim 1, Zitvogel discloses a method of treating or preventing cancer in a subject, comprising modulating levels of one or more commensal microbes (balance the gut microbiota; paragraph [0045]) within the subject to: (A) enhance an immune response by the subject (stimulating the body's immune system; paragraph [0033]), (B) inhibit the growth or spread of the cancer (tumor growth inhibition; paragraph [0098], (C) inhibit immune evasion by the cancer (CTX-induced anticancer immune response and tumor mass reduction; paragraph [0110], and/or (D) enhance the efficacy of a therapeutic (Gram-positive bacteria appear to be necessary for the optimal efficacy; paragraph [0110].

As per claim 2, Zitvogel discloses the method of claim 1, Zitvogel further discloses wherein the levels of one or more commensal microbes are modulated within the gut of the subject (balance the gut microbiota; paragraph [0045]).

As per claim 3, Zitvogel discloses the method of claim 1, Zitvogel further discloses wherein modulating the levels of one or more commensal microbes comprises increasing and/or decreasing levels of one or more bacterial selected from the genera Adlercreutzia, Oscillopira, Mollicutes, Butyrivibrio, Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidobacterium, Rikenella, Alistipes, Marinilabilia, Anaerostipes, Escherichia, and/or Lactobacillus (a probiotic bacterial strain, Lactobacillus johnsonii; paragraph [0057]).

As per claim 4, Zitvogel discloses the method of claim 1, Zitvogel further discloses wherein modulating the levels of one or more commensal microbes comprises administering a beneficial microbes to the subject (a probiotic bacterial strain, Lactobacillus johnsonii; paragraph [0057]).

As per claim 5, Zitvogel discloses the method of claim 4, Zitvogel further discloses wherein the beneficial microbes are bacteria (a probiotic bacterial strain, Lactobacillus johnsonii; paragraph [0057]).

As per claim 6, Zitvogel discloses the method of claim 5, Zitvogel further discloses wherein the bacteria are Lactobacillus (a probiotic bacterial strain, Lactobacillus johnsonii; paragraph [0057]).

As per claim 7, Zitvogel discloses the method of claim 6, and Zitvogel further discloses wherein the bacteria are Bifidobacterium (beneficial or "favorable" bacteria are essentially Lactobacillus and Bifidobacterium; paragraph [0027]).

As per claim 9, Zitvogel discloses the method of claim 4, Zitvogel discloses wherein the beneficial microbes are administered as a probiotic composition or via microflora transplant from a donor (to administer the probiotic; paragraph [0061]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 74, 86 and 94 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 74 and 86 are unclear for the following reason(s): Claims 74 and 86 state "wherein the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-All10, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT 01 1, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010". On page 6, line 26-30, they state "the immune checkpoint inhibitor is selected from the group consisting of nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-All10, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT 011, MPDL3280A, MEDI-4736, MSB-0020718C, 30 AUR-012 and STI-A1010". For examination purposes, "wherein the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-All10, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT 01 1, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010" will be considered as state "the immune checkpoint inhibitor is selected from the group consisting of nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-All10, TSR-042, RG-7446, BMS- 936559, BMS-936558, MK-3475, CT 011, MPDL3280A, MEDI-4736, MSB-0020718C, 30 AUR-012 or STI-A1010".

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Box V: Citations and Explanations-***-

As per claim 13, Zitvogel does not disclose the method of claim 12, Zitvogel does disclose further comprising administration of beneficial microbes to the subject (compensated with appropriate probiotics; paragraph [0046]).

As per claim 14, Zitvogel discloses the method of claim 1, Zitvogel also discloses further comprising administering to the subject a cancer therapy (used in combination with a chemotherapeutic agent; paragraph [0062].

As per claim 15, Zitvogel disclose the method of claim 14, Zitvogel further discloses wherein the modulating levels of one or more commensal microbes (balance the gut microbiota; paragraph [0045]) within the subject enhances an immune response by the subject (stimulating the body's immune system; paragraph [0033] and/or inhibits immune evasion by the cancer, and the cancer therapy is an immunotherapy (an biological immunotherapy; paragraph [0062]).

As per claim 16, Zitvogel discloses the method of claim 15, Zitvogel further discloses wherein the immunotherapy comprises administration of anti-CTLA-4 antibodies and/or anti-PD-L1 or anti-PD-1 antibodies (an anti-CTLA4 antibody, an anti-PD-1/PDL-1 Ab; paragraph [0056]).

As per claim 17, Zitvogel discloses the method of claim 14, Zitvogel further discloses wherein the modulating levels of one or more commensal microbes within the subject enhance the efficacy of a therapeutic, and the cancer therapy is said therapeutic (Thus, Gram-positive bacteria appear to be necessary for the optimal efficacy of the CTX-induced anticancer immune response and tumor mass reduction; paragraph [0110]).

As per claim 18, Zitvogel discloses the method of claim 17, Zitvogel further discloses wherein said therapeutic comprises a chemotherapeutic (CTX-based chemotherapy; paragraph [0109]).

As per claim 19, Zitvogel discloses the method of claim 1, Zitvogel also discloses further comprising testing the subject for immune evasion by the cancer (against which the CD4+ T cell response is analyzed; paragraph [0011]).

As per claim 20, Zitvogel discloses the method of claim 1, Zitvogel also discloses further comprising surgical, radiation, and/or chemotherapeutic cancer intervention (three cycles of chemotherapy; paragraph [0046].

As per claim 21, Zitvogel discloses a kit or composition comprising a beneficial commensal microbe and a cancer therapeutic, said composition or components of said kit formulated for therapeutic delivery to a subject (the present invention relates to the use of probiotics, in combination with an antineoplastic treatment, for treating a cancer patient; paragraph [0060]).

As per claim 22, Zitvogel discloses a beneficial commensal microbe for use as a medicament in the treatment of cancer and/or inhibition of immune evasion (to improve the efficacy of such a treatment; paragraph [0001]).

As per claim 23, Zitvogel discloses a method of treating or preventing cancer in a subject comprising administering to the subject bacterial formulation comprising bacteria of the genera Bifidobacterium (the "favorable" bacteria can be those from a group comprising or consisting of the genera Lactobacillus and Bifidobacterium; paragraph [0053]).

As per claim 26, Zitvogel discloses the method of claim 23, Zitvogel further discloses wherein the bacterial formulation comprise bacteria of the genus Bifidobacterium (the "favorable" bacteria can be those from a group comprising or consisting of the genera Lactobacillus and Bifidobacterium; paragraph [0053]).

As per claim 33, Zitvogel discloses the method of claim 26, Zitvogel further discloses wherein the bacterial formulation is administered by oral administration (the probiotic bacterial strain according to the invention is formulated for oral administration; paragraph [0059]).

As per claim 34, Zitvogel discloses the method of claim 27, Zitvogel further discloses wherein the bacterial formulation is a food product (as functional food such as drinks, fermented yoghurts; paragraph [0059]).

As per claim 57, Zitvogel discloses a method of treating cancer in a human subject comprising administering to the subject an immune checkpoint inhibitor (with an anti-CTLA4 antibody; paragraph [0056] and a bacterial formulation comprising bacteria of the genera Bifidobacterium (bacteria from a first group comprising Lactobacillus and Bifidobacterium genera; paragraph [0013]).

As per claim 61, Zitvogel discloses the method of claim 57, Zitvogel further discloses wherein the bacterial formulation is administered by oral administration or rectal administration (the probiotic bacterial strain according to the invention is formulated for oral administration; paragraph [0059]).

As per claim 62, Zitvogel discloses the method of claim 61, Zitvogel further discloses wherein the bacterial formulation is administered by oral administration (the probiotic bacterial strain according to the invention is formulated for oral administration; paragraph [0059]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
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Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claim 64, Zitvogel discloses the method of claim 1, Zitvogel further discloses wherein the bacterial formulation is administered to the subject in two or more doses (the probiotic strain can be administered prior to the antineoplastic agent (e. g., 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before; paragraph [0061]).

As per claim 65, Zitvogel discloses the method of claim 64, Zitvogel further discloses wherein the administration of the two or more doses are separated by at least 1 week (e. g., 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before; paragraph [0061]).

As per claim 68, Zitvogel discloses the method of claim 57, Zitvogel further discloses wherein the immune checkpoint inhibitor is a protein or polypeptide that binds to an immune checkpoint protein (with an anti-CTLA4 antibody; paragraph [0056]).

As per claim 69, Zitvogel discloses the method of claim 68, Zitvogel further discloses wherein the immune checkpoint protein is CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA (with an anti-CTLA4 antibody; paragraph [0056]) .

As per claim 70, Zitvogel discloses the method of claim 69, Zitvogel further discloses wherein the immune checkpoint protein is PD-1 or PD-LI (with an anti-CTLA4 antibody, an anti-PD-1/PDL-1 Ab; paragraph [0056]).

As per claim 71, Zitvogel discloses the method of claim 57, Zitvogel further discloses wherein the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to an immune checkpoint protein (with an anti-CTLA4 antibody, an anti-PD-1/PDL-1 Ab; paragraph [0056]).

As per claim 72, Zitvogel discloses the method of claim 71, Zitvogel further discloses wherein the immune checkpoint protein is CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA (with an anti-CTLA4 antibody, an anti-PD-1/PDL-1 Ab; paragraph [0056]).

As per claim 73, Zitvogel discloses the method of claim 72, Zitvogel further discloses wherein the immune checkpoint protein is PD-1 or PD-LI (with an anti-CTLA4 antibody, an anti-PD-1/PDL-1 Ab; paragraph [0056]).

As per claim 75, Zitvogel discloses the method of claim 57, Zitvogel further discloses wherein the immune checkpoint inhibitor is administered by intravenous injection, intramuscular injection, intratumoral injection or subcutaneous injection (CTLA4 blockade elicited pTh17 cells in the spleen after 3 injections; Figure 19C).

As per claim 79, Zitvogel discloses the method of claim 76, Zitvogel further discloses wherein the bacterial formulation is administered by oral administration or rectal administration (the probiotic bacterial strain according to the invention is formulated for oral administration; paragraph [0059]).

As per claim 80, Zitvogel discloses the method of claim 79, Zitvogel further discloses wherein the bacterial formulation is administered by oral administration (the probiotic bacterial strain according to the invention is formulated for oral administration; paragraph [0059]).

As per claim 81, Zitvogel discloses the method of claim 76, Zitvogel further discloses wherein the bacterial formulation is administered to the subject in two or more doses (the probiotic strain can be administered prior to the antineoplastic agent (e. g., 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before; paragraph [0061]).

As per claim 83, Zitvogel discloses the method of claim 76, Zitvogel also discloses further comprising administering to the subject an immune checkpoint inhibitor (with an anti-CTLA4 antibody; paragraph [0056]).

As per claim 84, Zitvogel discloses the method of claim 83, Zitvogel further discloses wherein the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA(with an anti-CTLA4 antibody; paragraph [0056]).

As per claim 85, Zitvogel discloses the method of claim 83, Zitvogel further discloses wherein the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to PD-1 or PD-LI (with an anti-CTLA4 antibody, an anti-PD-1/PDL-1 Ab; paragraph [0056]).

As per claim 88, Zitvogel discloses the method of claim 87, Zitvogel also discloses further comprising administering to the recipient subject an immune checkpoint inhibitor (with an anti-CTLA4 antibody; paragraph [0056]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claim 89, Zitvogel discloses the method of claim 88, Zitvogel further discloses wherein the immune checkpoint inhibitor is a protein or polypeptide that specifically binds to an immune checkpoint protein (with an anti-CTLA4 antibody; paragraph [0056]).

As per claim 90, Zitvogel discloses the method of claim 88, Zitvogel further discloses wherein the immune checkpoint protein is selected from the group consisting of CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA (with an anti-CTLA4 antibody; paragraph [0056]).

As per claim 91, Zitvogel discloses the method of claim 89 or 90. Zitvogel further discloses wherein the polypeptide or protein is an antibody or antigen-binding fragment thereof (with an anti-CTLA4 antibody; paragraph [0056]).

Claims 10-12 lack an inventive step under PCT Article 33(3) as being obvious over EP 2 876 167 A1 to Zitvogel in view of the article "Cancer and the gut microbiota: An unexpected link" Zitvogel, et al, (hereinafter "Zitvogel'15").

As per claim 10, Zitvogel discloses the method of claim 1, Zitvogel does not disclose in EP2876167A1 wherein modulating the levels of one or more commensal microbes comprises administering one or more antimicrobials. However, Zitvogel'15 does disclose modulating the levels of one or more commensal microbes comprises administering one or more antimicrobials; Using common antibiotics; page 3, column 2, paragraph 2, line 1). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to include an antimicrobial as taught by Zitvogel'15 in order to improve the clinical efficacy of the anticancer therapy.

As per claim 11, Zitvogel does not disclose the method of claim 10, Zitvogel also does not disclose wherein the antimicrobial kills detrimental microbes in EP2876167A1. However, Zitvogel'15 discloses wherein the antimicrobial kills detrimental microbes (it may be possible to use antibiotics to reverse a previously established state of detrimental dysbiosis; page 3, column 2, paragraph 2, lines 7-9).). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to add an antimicrobial as taught by Zitvogel'15 in order to improve the clinical efficacy of the anticancer therapy.

As per claim 12, Zitvogel does not disclose the method of claim 11, Zitvogel does not disclose wherein the antimicrobial is an antibiotic. However, Zitvogel'15 does disclose the method of claim 11 and further discloses wherein the antimicrobial is an antibiotic (it may be possible to use antibiotics to reverse a previously established state of detrimental dysbiosis; page 3, column 2, paragraph 2, lines 7-9). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to an antibiotic as taught by Zitvogel'15 in order to improve the clinical efficacy of the anticancer therapy.

Claims 8, 24-25, 27-28, 29/26-28, 58-60, 63, and 76-78 lack an inventive step under PCT Article 33(3) as being obvious over Zitvogel in view of US 7,195,906 B2 to Collins, et al. (hereinafter "Collins").

As per claim 24, Zitvogel discloses the method of claim 23, Zitvogel does not disclose wherein at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, Rikenella, Alistipes, Marini lab ilia, or Anaerostipes. However, Collins does disclose wherein at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, Rikenella, Alistipes, Marini lab ilia, or Anaerostipes (provides a formulation which comprises a strain of Bifidobacterium; column 4 lines 11-12). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 25, Zitvogel discloses the method of claim 23, Zitvogel does not disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, Rikenella, Alistipes, Marini lab ilia, or Anaerostipes. However, Collins does disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, Rikenella, Alistipes, Marini lab ilia, or Anaerostipes (provides a formulation which comprises a strain of Bifidobacterium; column 4 lines 11-12). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 27, Zitvogel discloses the method of claim 26, Zitvogel does not disclose wherein at least 50% of the bacteria in the bacterial formulation are of the genus Bifidobacterium. . However, Collins does disclose wherein at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, Rikenella, Alistipes, Marini lab ilia, or Anaerostipes (provides a formulation which comprises a strain of Bifidobacterium; column 4 lines 11-12). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 28, Zitvogel discloses the method of claim 26, Zitvogel does not disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genus Bifidobacterium. However, Collins does disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genus Bifidobacterium (provides a formulation which comprises a strain of Bifidobacterium; column 4 lines 11-12). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Supplemental Box

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As per claim 58, Zitvogel discloses the method of claim 57, Zitvogel does not disclose wherein at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium. However, Collins does disclose wherein at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium (provides a formulation which comprises a strain of Bifidobacterium; column 4 lines 11-12). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 59, Zitvogel discloses the method of claim 57, Zitvogel does not disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genera Bifidobacterium. However, Collins does disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genera Bifidobacterium (provides a formulation which comprises a strain of Bifidobacterium; column 4 lines 11-12). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claims 8, 29/26, and 60, Zitvogel discloses the method of claim 7, 26, and 57; but Zitvogel does not disclose wherein the Bifidobacterium include bacteria selected from the group consisting of Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, and Bifidobacterium angulatum. However, Collins discloses wherein the Bifidobacterium longum and Bifidobacterium infantis (a naturally occurring variant of Bifidobacterium longum infantis UCC35624; column 4, lines 1-5). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to include wherein the Bifidobacterium include bacteria is Bifidobacterium longum, and/or Bifidobacterium infantis, as taught by Collins, in order to provide a probiotic containing well known beneficial organisms.

As per claims 29/27-28, Zitvogel and Collins, in combination, disclose the methods of claim 27-28; but Zitvogel does not disclose wherein the Bifidobacterium include bacteria selected from the group consisting of Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, and Bifidobacterium angulatum. However, Collins discloses wherein the Bifidobacterium longum and Bifidobacterium infantis (a naturally occurring variant of Bifidobacterium longum infantis UCC35624; column 4, lines 1-5). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to include wherein the Bifidobacterium include bacteria is Bifidobacterium longum, and/or Bifidobacterium infantis, as taught by Collins, in order to provide a probiotic containing well known beneficial organisms.

As per claim 63, Zitvogel discloses the method of claim 57, Zitvogel does not disclose wherein the bacterial formulation comprises at least 5x106 CFU of bacteria of the genera Bifidobacterium. However, Collins does disclose wherein the bacterial formulation comprises at least 5x106 CFU of bacteria of the genera Bifidobacterium (the Bifidobaclerium is present at more than 106 cfu per gram; column 4, lines 64-65). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 76, Zitvogel discloses a method of treating cancer in a human subject comprising administering to the subject a bacterial formulation (the "favorable" bacteria can be those from a group comprising or consisting of the genera Lactobacillus and Bifidobacterium; paragraph [0053]). Zitvogel does not disclose wherein comprising at least 5x106 CFU of bacteria of the genera Bifidobacterium, wherein at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium. However, Collins does disclose wherein the bacterial formulation comprises at least 5x106 CFU of bacteria of the genera Bifidobacterium (the Bifidobaclerium is present at more than 106 cfu per gram; column 4, lines 64-65). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 77, Zitvogel and Collins, in combination, disclose the method of claim 76, but Zitvogel does not disclose wherein at least 90% of the bacteria in the bacterial formulation are of the genera Bifidobacterium. However, Collins does disclose wherein the bacterial formulation comprises at least 5x106 CFU of bacteria of the genera Bifidobacterium (the Bifidobaclerium is present at more than 106 cfu per gram; column 4, lines 64-65). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to be at least 50% Bifidobacterium as taught by Collins as this is a standard industry formulation (Align Probiotic Supplement).

As per claim 78, Zitvogel and Collins, in combination, disclose the method of claim 76; but Zitvogel does not disclose wherein the Bifidobacterium include bacteria selected from the group consisting of Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, and Bifidobacterium angulatum. However, Collins discloses wherein the Bifidobacterium longum and Bifidobacterium infantis (a naturally occurring variant of Bifidobacterium longum infantis UCC35624; column 4, lines 1-5). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to include wherein the Bifidobacterium include bacteria is Bifidobacterium longum, and/or Bifidobacterium infantis, as taught by Collins, in order to provide a probiotic containing well known beneficial organisms.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Supplemental Box

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Claims 66-67, 87, 111-112, and 113/111-112 lack an inventive step under PCT Article 33(3) as being obvious over Zitvogel in view of WO 2014/145958 A4 to Seres Health, Inc. (hereinafter, "Henn").

As per claim 66, Zitvogel discloses the method of claim 57, Zitvogel does not disclose further comprising administering to the subject an antibiotic prior to the administration of the bacterial formulation. However, Henn does disclose further comprising administering to the subject an antibiotic prior to the administration of the bacterial formulation (one way of preparing the patient for administration of the microbial ecosystem, at least one antibiotic can be administered; paragraph [0187]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to pretreat the subject with an antibiotic as taught by Henn in order to improve the clinical efficacy of the anticancer therapy.

As per claim 67, Zitvogel discloses the method of claim 66, Zitvogel further discloses wherein the antibiotic is administered to the subject at least 1 day before the bacterial formulation is administered to the subject (the antibiotic can be discontinued 1, 2, or 3 days before the administration of the bacterial composition; paragraph [0188]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to pretreat the subject with an antibiotic as taught by Henn in order to improve the clinical efficacy of the anticancer therapy.

As per claim 87, Zitvogel discloses wherein the donor subject comprises microflora that promotes the treatment of cancer by immune checkpoint inhibitor immunotherapy (for use in combination with an antineoplastic agent for inducing a T-bet/Th1 local and systemic immune response, for treating a cancer; paragraph [0057]), Zitvogel does not disclose a method of treating or preventing cancer in a recipient subject, the method comprising administering to the recipient subject a bacterial formulation comprising microflora obtained from a donor subject, wherein the donor subject comprises microflora that promotes the treatment of cancer by immune checkpoint inhibitor immunotherapy. However, Henn discloses a method of treating or preventing cancer in a recipient subject, the method comprising administering to the recipient subject a bacterial formulation comprising microflora obtained from a donor subject (the therapeutic bacterial composition is substantially depleted of a residual habitat product of a fecal material. In certain aspects, the composition is formulated for oral administration; paragraph [0057]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to obtain microflora from a donor subject as taught by Henn in order to improve the clinical efficacy of the anticancer therapy.

As per claim 111, Zitvogel discloses wherein the donor subject comprises microflora that promotes the treatment of cancer by immune checkpoint inhibitor immunotherapy (for use in combination with an antineoplastic agent for inducing a T-bet/Th1 local and systemic immune response, for treating a cancer; paragraph [0057]). Zitvogel does not disclose a method of treating or preventing cancer in a recipient subject, the method comprising administering to the recipient subject a bacterial formulation comprising microflora obtained from a donor subject, wherein the donor subject comprises microflora that promotes the treatment of cancer by immune checkpoint inhibitor immunotherapy. However, Henn discloses a method of treating or preventing cancer in a recipient subject, the method comprising administering to the recipient subject a bacterial formulation comprising microflora obtained from a donor subject (the therapeutic bacterial composition is substantially depleted of a residual habitat product of a fecal material. In certain aspects, the composition is formulated for oral administration; paragraph [0057]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to obtain microflora from a donor subject as taught by Henn in order to improve the clinical efficacy of the anticancer therapy.

As per claim 112, Zitvogel and Henn, in combination, disclose the bacterial formulation of claim 111, but Zitvogel does not disclose fecal material of the donor subject. However, Henn discloses using fecal material of the donor subject (the therapeutic bacterial composition is substantially depleted of a residual habitat product of a fecal material; paragraph [0057]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to obtain microflora from fecal material of the donor subject, as taught by Henn, in order to improve the clinical efficacy of the anticancer therapy.

As per claims 113/111-112, Zitvogel and Henn, in combination, disclose the bacterial formulation of claims 111-112, but Zitvogel does not disclose wherein one or more detrimental microbes originally present in the microflora have been removed or killed (the therapeutic bacterial composition is substantially depleted of a residual habitat product of a fecal material; paragraph [0057]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to provide wherein one or more detrimental microbes originally present in the microflora have been removed or killed, as taught by Henn, in order to improve the clinical efficacy of the anticancer therapy by not introducing dangerous microorganisms into the patient.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/35228

Supplemental Box

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Claims 74, 86, and 94 lack an inventive step under PCT Article 33(3) as being obvious over Zitvogel in view of WO 2015/061372 A1 to Hemoshear, LLC (hereinafter "Wamhoff").

As per claims 74, 86, and 94, Zitvogel discloses the method of claim 57, 83, and 88; but Zitvogel does not disclose wherein the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-All10, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT 01 1, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010. However, Wamhoff discloses wherein the immune checkpoint inhibitor is nivolumab, pembrolizumab, or pidilizumab (immunotherapy comprises the targeted immunotherapy, the targeted immunotherapy comprising nivolumab, pidilizumab, or a combination thereof; claim 174). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to include any of the inhibitors from this list as taught by Wamhoff, because many of them are commercially available.

Claims 92 and 93 lack an inventive step under PCT Article 33(3) as being obvious over Zitvogel in view of WO 2011/068810 A1 to Shire Human Genetic Therapies (hereinafter, "Guild").

As per claim 92, Zitvogel discloses the method of claim 88, Zitvogel does not disclose wherein the immune checkpoint inhibitor is an interfering nucleic acid molecule. However, Guild discloses wherein the immune checkpoint inhibitor is an interfering nucleic acid molecule (suitable RNA includes mRNA, siRNA, miRNA, snRNA and snoRNA; page 8, line 32 – page 9, line 1). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to use an interfering nucleic acid as taught by Guild in order to improve the clinical efficacy of the anticancer therapy.

As per claim 93, Zitvogel and Guild, in combination, disclose the method of claim 92, but Zitvogel does not disclose wherein the interfering nucleic acid molecule is an siRNA molecule, an shRNA molecule or an antisense RNA molecule. However, Guild discloses wherein the interfering nucleic acid molecule is an siRNA molecule, an shRNA molecule or an antisense RNA molecule (suitable RNA includes mRNA, siRNA, miRNA, snRNA and snoRNA; page 8, line 32 – page 9, line 1). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to use an interfering nucleic acid as taught by Guild in order to improve the clinical efficacy of the anticancer therapy.

Claim 82 lacks an inventive step under PCT Article 33(3) as being obvious over Zitvogel in view of Collins and further in view of Henn.

As per claim 82, Zitvogel and Collins, in combination, disclose the method of claim 76, but Zitvogel does not disclose further comprising administering to the subject an antibiotic before the bacterial formulation is administered to the subject. However, Henn does disclose further comprising administering to the subject an antibiotic prior to the administration of the bacterial formulation (one way of preparing the patient for administration of the microbial ecosystem, at least one antibiotic can be administered; paragraph [0187]). It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the Zitvogel invention to pretreat the subject with an antibiotic as taught by Henn in order to improve the clinical efficacy of the anticancer therapy.

Claims 1-28, 29/26-28, 33-34, 57-90, 91/89-90, 92-94, and 111-112, 113/111-112 have industrial applicability as defined by PCT Article 33 (4) because the subject matter can be made or used in industry.

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/170,284	Filing Date 06/01/2016	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **02/06/2017**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0	X $40 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	***2	= 0	X $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
SHAUNA ZIMMERMAN

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/170,284	06/01/2016	Thomas F. Gajewski	UCHI-34458/US-3/ORD

CONFIRMATION NO. 8885

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

PUBLICATION NOTICE

OC00000087747835

Title:TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA

Publication No.US-2016-0354416-A1
Publication Date:12/08/2016

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/170,284	06/01/2016	Thomas F. Gajewski	UCHI-34458/US-3/ORD	8885

72960 7590 11/04/2016

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EXAMINER
HINES, JANA A

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
11/04/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

docketing@casimirjones.com
pto.correspondence@casimirjones.com

Office Action Summary	Application No. 15/170,284	Applicant(s) GAJEWSKI ET AL.	
	Examiner Ja'Na Hines	Art Unit 1645	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *September 1, 2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-30* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-30* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Claim Status

1. Claims 1-30 are under consideration in this Office Action.

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

2. Claims 1-30 are rejected under 35 U.S.C. 103 as being unpatentable over Sharon et al., (Chin. J.Cancer. 2014. 33(9):434-444) in view of O'Mahoney et al., (US Patent Application 2012/0276143 published Nov. 2012).

The claims are drawn to a method of treating cancer in a human subject comprising administering to the subject an immune checkpoint inhibitor and a bacterial formulation comprising bacteria of the genera Bifidobacterium.

Sharon et al., teach immunology-based therapy is rapidly developing into an effective treatment option for a surprising range of cancers (abstract). Sharon et al., learned over the last decade that powerful immunologic effector cells may be blocked by inhibitory regulatory pathways controlled by specific molecules often called "immune checkpoints." (page 434). These checkpoints serve to control or turn off the immune response when it is no longer needed to prevent tissue injury and autoimmunity. Cancer cells have learned or evolved to use these mechanisms to evade immune control and elimination. The development of a new therapeutic class of drugs that inhibit these inhibitory pathways has recently emerged as a potent strategy in oncology. Three sets of agents have emerged in clinical trials exploiting this strategy. These agents are antibody-based therapies targeting cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed cell death 1 (PD-1), and programmed cell death ligand 1 (PD-L1)(page 434). See Table 1 for additional immune checkpoint proteins and their inhibitors, such

as B7H3, Lag3, and KIR (page 435). Sharon et al., teach CTLA4 inhibition, human cancers and the inhibitor being an antibody (page 436). These inhibitors of immune inhibition have demonstrated extensive activity as single agents and in combinations. Clinical responses have been seen in melanoma, renal cell carcinoma, non-small cell lung cancer, urothelial, head and neck, ovarian cancer and various lymphomas (page 434). Sharon et al., teach PD-1 and PDL1 targeting agents (pages 439-440). Despite the autoimmune or inflammatory immune-mediated adverse effects which have been seen, the responses and overall survival benefits exhibited thus far warrant further clinical development (page 434). Sharon et al., a method of treating cancer in a human subject comprising administering to the subject an immune checkpoint inhibitor.

O'Mahoney et al., teach *Bifidobacterium* strain which has been shown to have immunomodulatory effects, by modulating cytokine levels or by antagonizing and excluding pro-inflammatory micro-organisms from the gastrointestinal tract [para. 0005]. O'Mahoney et al., teach *Bifidobacterium* strain may be significantly immunomodulatory following oral consumption in human [para. 0007]. *Bifidobacterium* strain or a formulation as described herein for use in the prophylaxis and/or treatment of gastrointestinal cancer(s) [para. 0013]. *Bifidobacterium* strain or a formulation as described herein for use in the prophylaxis and/or treatment of cancer due to undesirable inflammatory activity [para. 0016]. O'Mahoney et al., teach a variety of administration protocols including daily [para. 0128] The combined administration of a probiotic strain with one or more prebiotic compounds may enhance the growth of the administered probiotic in vivo resulting in a more pronounced health benefit, and is

termed symbiotic [para. 0139]. It will be appreciated that the probiotic strains may be administered prophylactically or as a method of treatment either on its own or with other probiotic and/or prebiotic materials as described above. In addition, the bacteria may be used as part of a prophylactic or treatment regime using other active materials such as those used for treating inflammation or other disorders especially those with an immunological involvement. Such combinations may be administered in a single formulation or as separate formulations administered at the same or different times and using the same or different routes of administration [para. 0140]. The *Bifidobacterium* strain may be present in an amount of more than 10^6 cfu per gram of the formulation [para. 0008]. The formulation may further comprise a drug entity. The formulation may further comprise a biological compound. The formulation may be used for immunization and vaccination protocols [para. 008].

Therefore, it would have been *prima facie* obvious at the time of applicants' invention to incorporate O'Mahoney's Bifidobacterium to Sharon's method for treating cancer in a human subject comprising administering to the subject an immune checkpoint inhibitor when O'Mahoney et al., already it was known to treat cancer in a human subject comprising administering to the subject Bifidobacterium in combination with other therapies that also treat cancer. One of ordinary skill in the art would have a reasonable expectation of success by combining both components because the prior art combination therapy was well known to produce beneficial and even synergistic results.

It is noted, that while the references recite oral administration at the instantly claimed dosage amount, there is no specific teaching of the dosage routines and

previous administration routines as recited by claims 8-11 and 25-26. Regarding the specific schemes recited in the instant claims, MPEP 2144.05 states, "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955) (Claimed process which was performed at a temperature between 40℃ and 80℃ and an acid concentration between 25% and 70% was held to be *prima facie* obvious over a reference process which differed from the claims only in that the reference process was performed at a temperature of 100℃ and an acid concentration of 10%.); see also *Peterson*, 315 F.3d at 1330, 65 USPQ2d at 1382 ("The normal desire of scientists or artisans to improve upon what is already generally known provides the motivation to determine where in a disclosed set of percentage ranges is the optimum combination of percentages."); *In re Hoeschele*, 406 F.2d 1403, 160 USPQ 809 (CCPA 1969) (Claimed elastomeric polyurethanes which fell within the broad scope of the references were held to be unpatentable thereover because, among other reasons, there was no evidence of the criticality of the claimed ranges of molecular weight or molar proportions.). For more recent cases applying this principle, see *Merck & Co. Inc. v. Biocraft Laboratories Inc.*, 874 F.2d 804, 10 USPQ2d 1843 (Fed. Cir.), *cert. denied*, 493 U.S. 975 (1989); *In re Kulling*, 897 F.2d 1147, 14 USPQ2d 1056 (Fed. Cir. 1990); and *In re Geisler,* 116 F.3d 1465, 43 USPQ2d 1362 (Fed. Cir. 1997)."

Additionally, *KSR International Co. v. Teleflex Inc.,* 127 S. Ct. 1727, 1741 (2007), discloses combining prior art elements according to known methods to yield predictable results, thus the combination is obvious unless its application is beyond that person's skill. *KSR International Co. v. Teleflex Inc.,* 127 S. Ct. 1727, 1741 (2007) also discloses that "The combination of familiar element according to known methods is likely to be obvious when it does no more than yield predictable results". It is well known to take a method of treating cancer when each ingredient is well-known to treat cancer, and where there is no change in the respective function of inhibitor or the *Bifidobacterium*; thus the combination would have yielded a reasonable expectation or success along with predictable results to one of ordinary skill in the art at the time of the invention. Therefore, it would have been obvious to a person of ordinary skill in the art to combine prior art elements according to known methods that is ready for improvement to yield predictable results. The claimed invention is *prima facie* obvious in view of the teachings of the prior art, absent any convincing evidence to the contrary.

Conclusion

3. No allowed claims.

4. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ja-Na Hines whose telephone number is 571-272-0859. The examiner can normally be reached Monday thru Thursday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Gary Nickol, can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Ja'Na Hines/
Primary Examiner, Art Unit 1645

Notice of References Cited	Application/Control No. 15/170,284	Applicant(s)/Patent Under Reexamination GAJEWSKI ET AL.	
	Examiner Ja'Na Hines	Art Unit 1645	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-2012/0276143 A1	11-2012	O'MAHONY; Liam	A23C9/1234	424/234.1
	B	US-				
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Sharon et al., (Chin. J.Cancer. 2014. 33(9):434-444)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15170284	GAJEWSKI ET AL.
	Examiner	Art Unit
	JA'NA HINES	1645

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
searched inventors, applications, patents. Commerical database search of claim text	10/2016	jah

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

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Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON WI 53562

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 15/170,284

1. THE REQUEST FILED June 1, 2016 IS **GRANTED.**

The above-identified application has met the requirements for prioritized examination

A. ☒ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;
B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
C. filing a **request for continued examination**;
D. filing a notice of appeal;
E. filing a request for suspension of action;
F. mailing of a notice of allowance;
G. mailing of a final Office action;
H. completion of examination as defined in 37 CFR 41.102; or
I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Brian W. Brown at 571-272-5338.

/Brian W. Brown/
[*Signature*]

Petitions Examiner, Office of Petitions
(Title)

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/170,284

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	30 minus 20 =	* 10	x 40 =	400	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*	x 210 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	1130		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/170,284	06/01/2016	1653	1200	UCHI-34458/US-3/ORD	30	2

CONFIRMATION NO. 8885

UPDATED FILING RECEIPT

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

OC000000085438488

Date Mailed: 08/30/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Thomas F. Gajewski, Chicago, IL;
Ayelet Sivan, Chicago, IL;
Leticia Corrales, Chicago, IL;

Applicant(s)

The University of Chicago, Chicago, IL;

Power of Attorney: The patent practitioners associated with Customer Number 72960

Domestic Priority data as claimed by applicant

This appln claims benefit of 62/169,112 06/01/2015
and claims benefit of 62/248,741 10/30/2015

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 06/15/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/170,284**

Projected Publication Date: 12/08/2016

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA

Preliminary Class

424

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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DocCode – SCORE

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Application Number: 15170284 Document Date: 08/17/2016

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- Drawing

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Form Revision Date: August 26, 2013

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	University of Chicago	Confirmation No.:	8885
Serial No.:	15/170,284	Art Unit:	TBD
Filed:	01-JUNE-2016	Examiner:	TBD
Title:	**TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA**		

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS MAILED JUNE 17, 2016

VIA EFS-WEB
COMMISSIONER FOR PATENTS
P.O. BOX 1450
ALEXANDRIA, VA 22313-1450

Sir or Madam:

This communication is responsive the Notice to File Corrected Application Papers mailed June 17, 2016.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: UCHI-34458/US-3/ORD. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

AMENDMENTS TO THE DRAWINGS

Please replace originally filed Figures 1-15 (38 sheets) with replacement Figures 1-15 (46 sheets) filed herewith.

REMARKS

In the Notice to File Corrected Application Papers mailed June 17, 2016, the Applicant was notified that Figures 1D, 2C, 3F, 7B, 8B, 9C, 12B, 13A, and 14A were not compliant with 37 C.F.R. 1.84 and 1.121(d). Applicant files herewith replacement Figures 1-15 (46 sheets) correcting the deficiencies referenced in the Notice. No new matter is added.

Fees

The Notice further indicates that the $70 Small Entity Surcharge is due. Applicant submits herewith $70 for the required fees.

Applicant believes no fees are due in connection with this filing. However, if the Director finds any additional fees to be due in connection with this, or any other filing, authorization is given to charge said fees to Deposit Account No. 50-4302, referencing attorney docket number UCHI-34572/US-3/CON.

Respectfully submitted,

Date: August 17, 2016

/David W. Staple/

David W. Staple
Registration No. 65,903
Casimir Jones S.C.
2275 Deming Way, Suite 310
Middleton, WI 53562
Phone: (608) 662-1277
Fax: (608) 662-1276

FIG. 1A

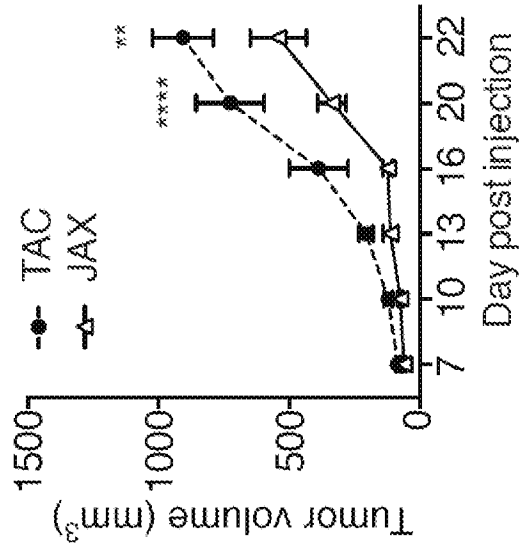


FIG. 1B

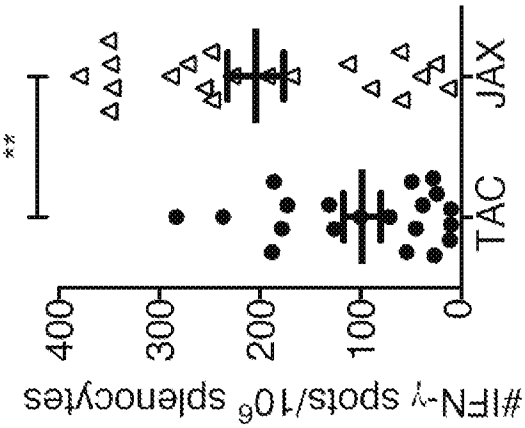


FIG. 1C

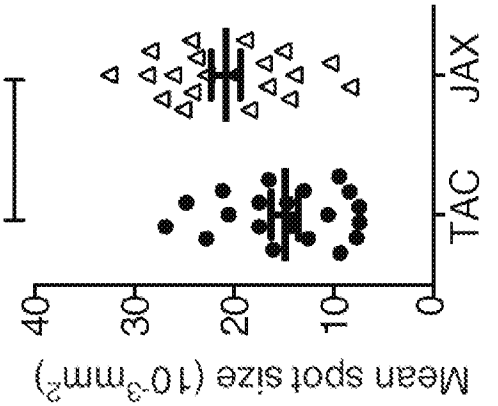


FIG. 1D

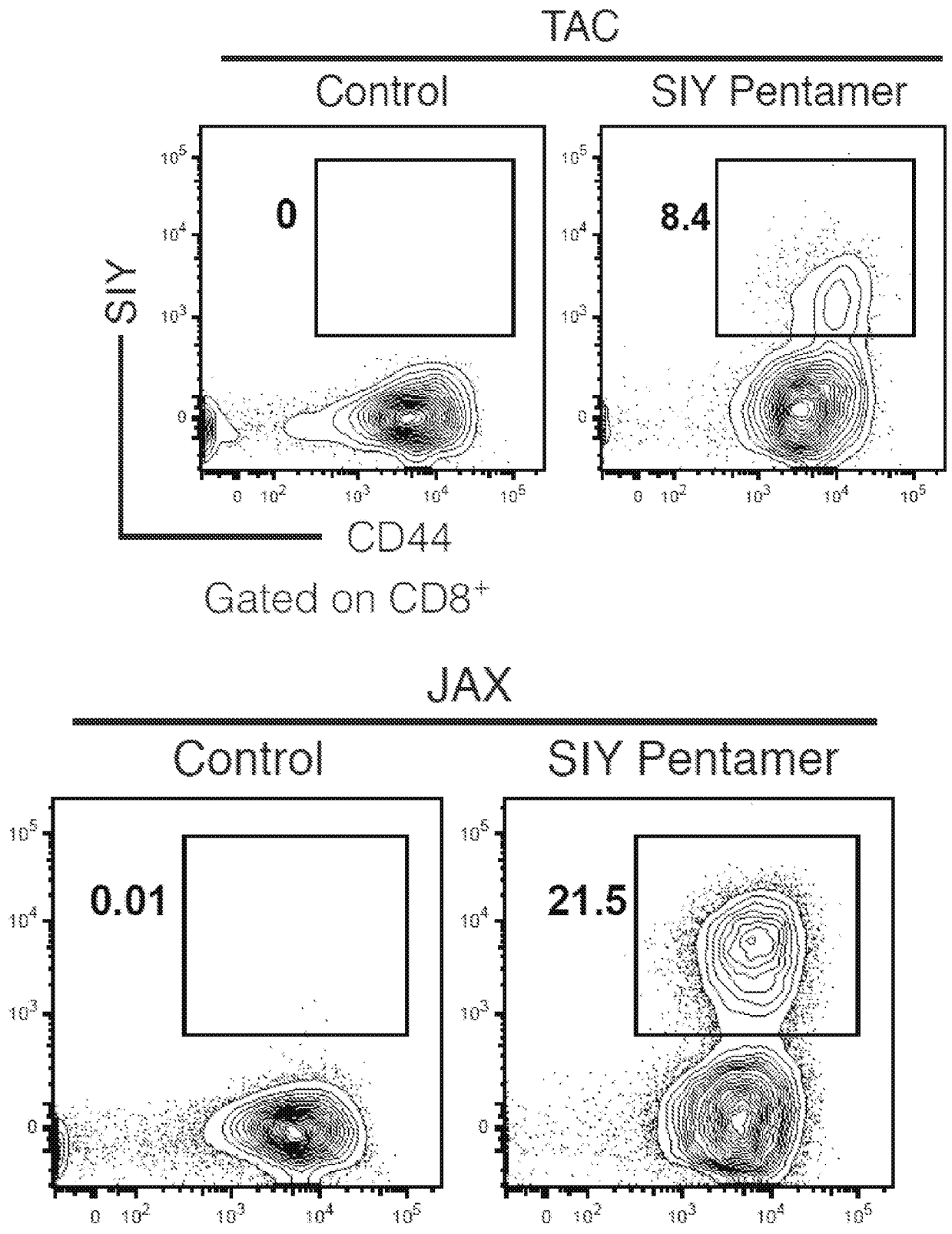
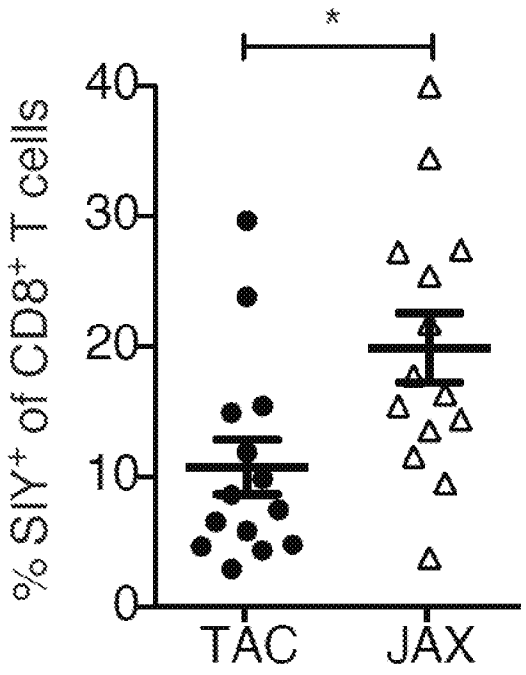

TAC
Control
SIY Pentamer
0
8.4
SIY
CD44
Gated on CD8+
JAX
Control
SIY Pentamer
0.01
21.5

FIG. 1D



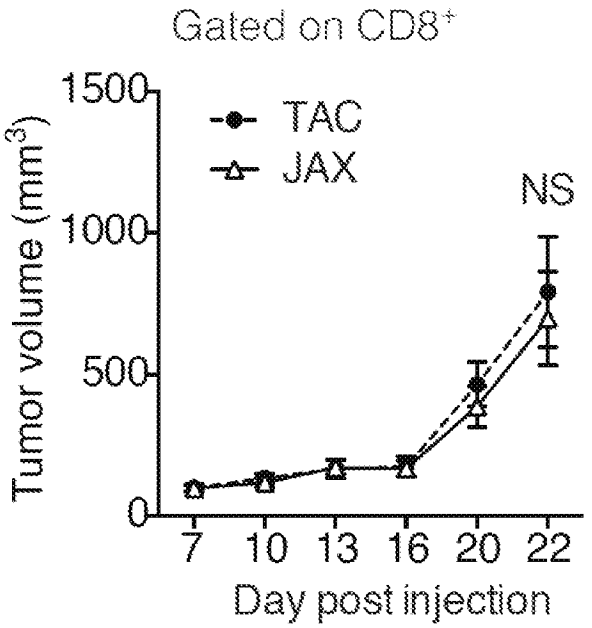


FIG. 1E

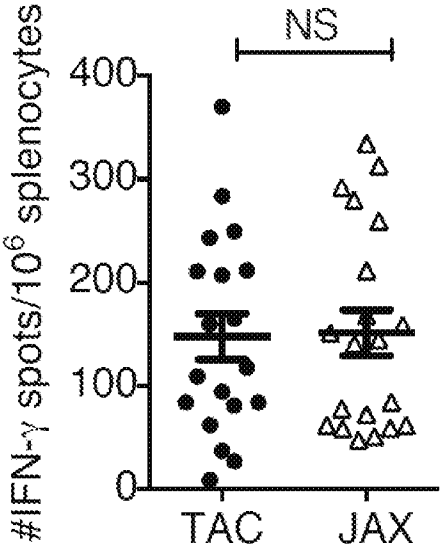


FIG. 1F

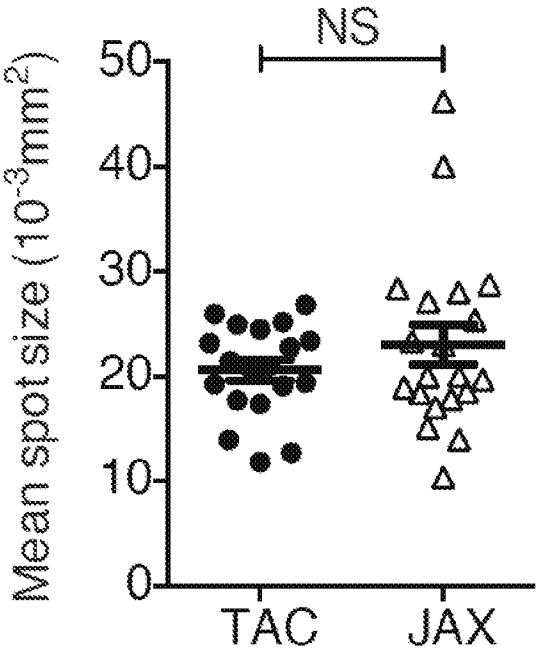


FIG. 1G

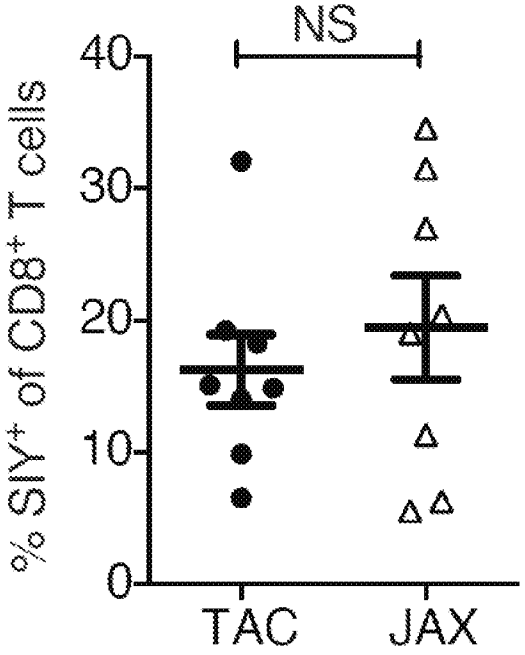


FIG. 1H

FIG. 2A

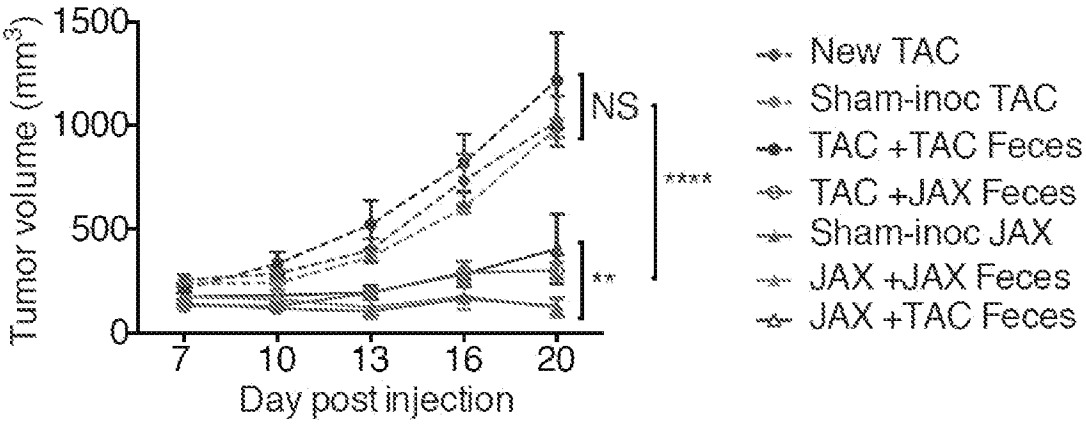

Tumor volume (mm3)
1500
1000
500
0
7
10
13
16
20
Day post injection
NS
****
**
New TAC
Sham-inoc TAC
TAC +TAC Feces
TAC +JAX Feces
Sham-inoc JAX
JAX +JAX Feces
JAX +TAC Feces

FIG. 2B

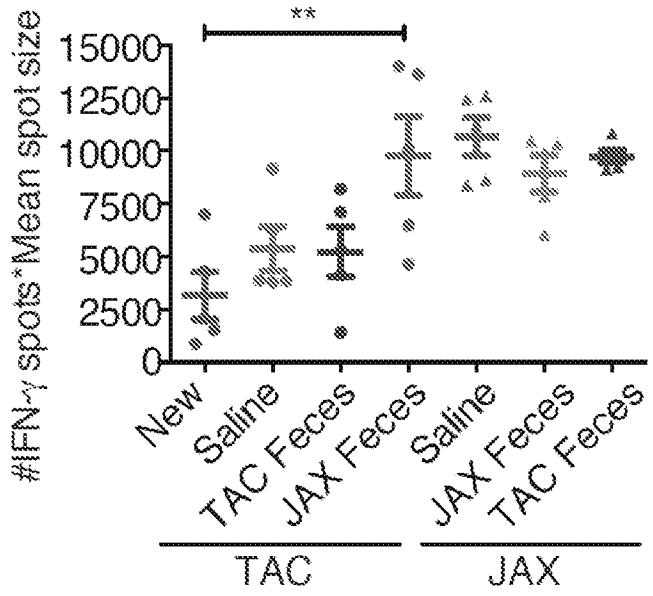

#IFN-γ spots*Mean spot size
15000
12500
10000
7500
5000
2500
0
**
New
Saline
TAC Feces
JAX Feces
Saline
JAX Feces
TAC Feces
TAC
JAX

FIG. 2C

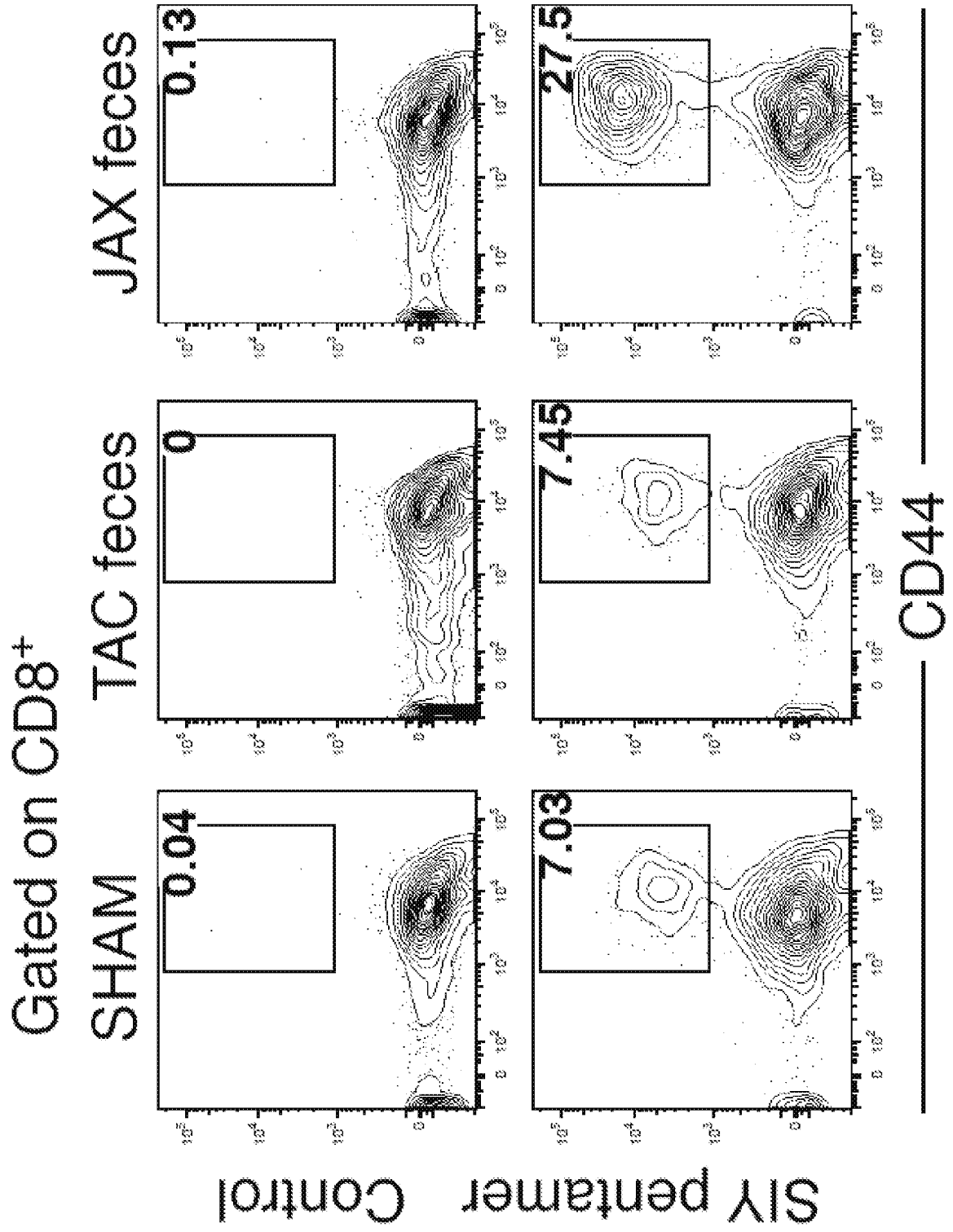

Gated on CD8+
SHAM
TAC feces
JAX feces
0.04
0
0.13
7.03
7.45
27.5
SIV pentamer Control
CD44

FIG. 2C (cont.)

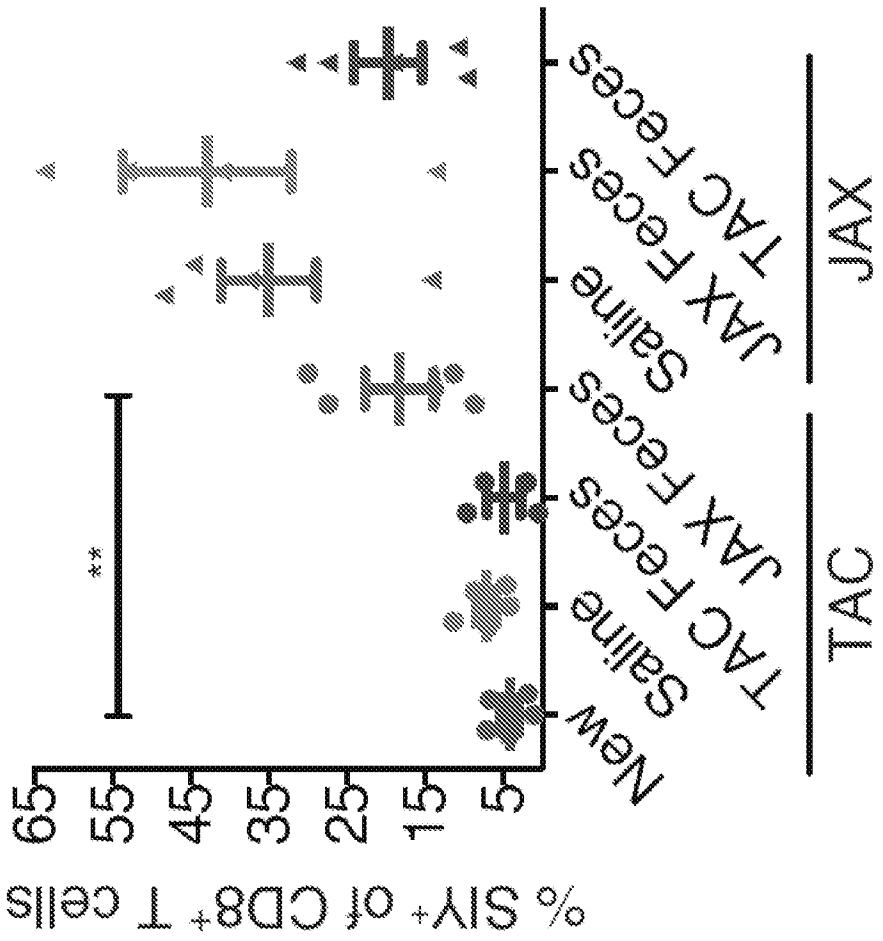

% SIV+ of CD8+ T cells
65
55
45
35
25
15
5
**
New
Saline
TAC Feces
JAX Feces
Saline
JAX Feces
TAC Feces
TAC
JAX

FIG. 2D

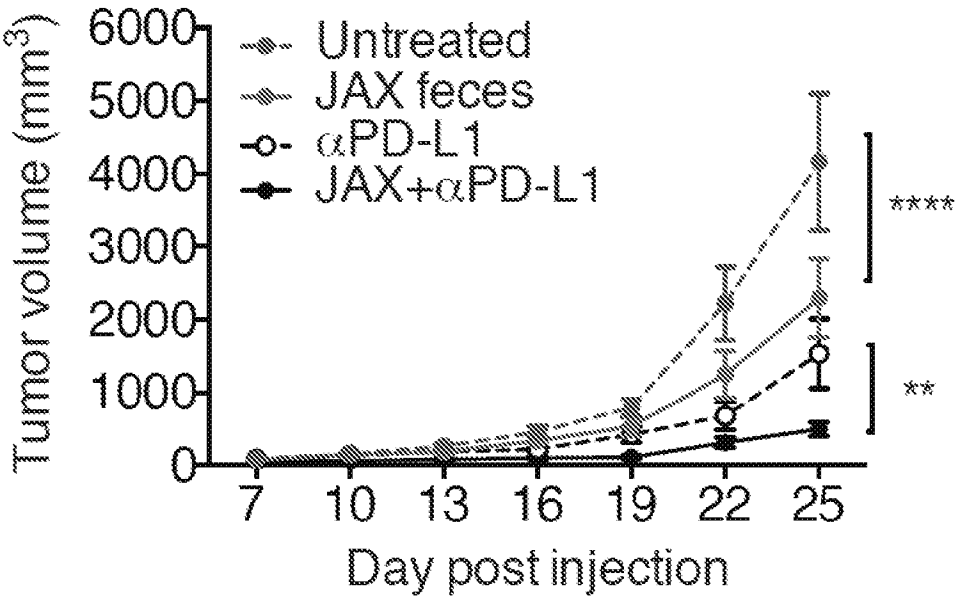

Tumor volume (mm3)
6000
5000
4000
3000
2000
1000
0
Untreated
JAX feces
αPD-L1
JAX+αPD-L1
****
**
7
10
13
16
19
22
25
Day post injection

FIG. 2E

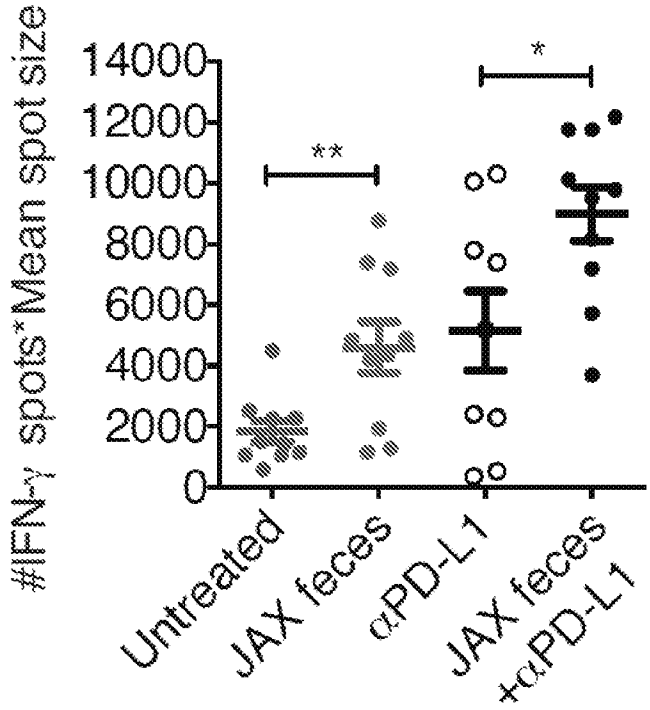

#IFN-γ spots*Mean spot size
14000
12000
10000
8000
6000
4000
2000
0
**
*
Untreated
JAX feces
αPD-L1
JAX feces
+αPD-L1

FIG. 2F

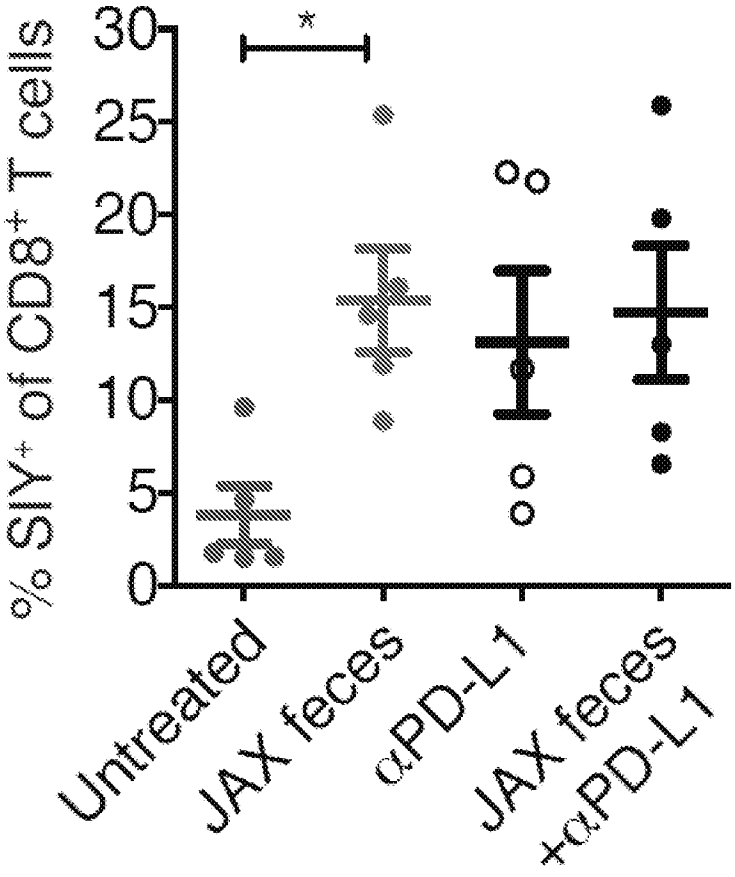

% SIY+ of CD8+ T cells
30
25
20
15
10
5
0
*
Untreated
JAX feces
αPD-L1
JAX feces
+αPD-L1

FIG. 2G

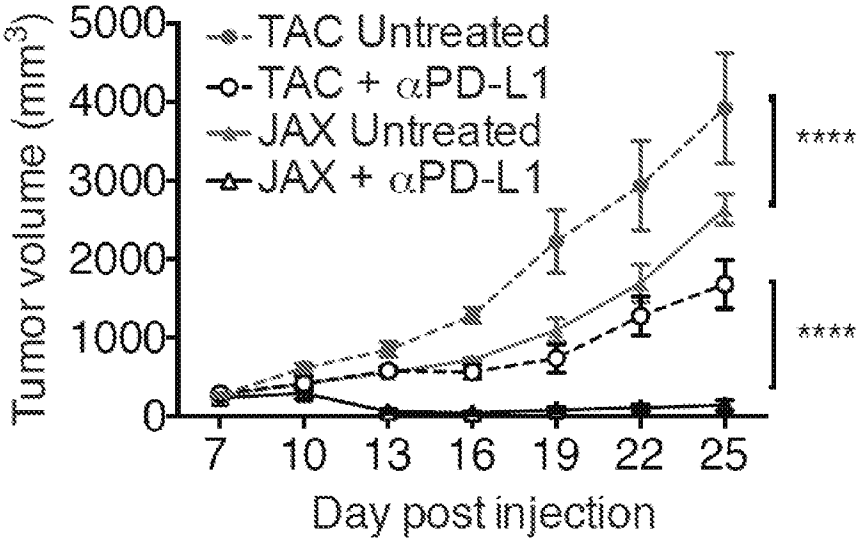

Tumor volume (mm3)
5000
4000
3000
2000
1000
0
TAC Untreated
TAC + αPD-L1
JAX Untreated
JAX + αPD-L1
****
****
7
10
13
16
19
22
25
Day post injection

FIG. 3A

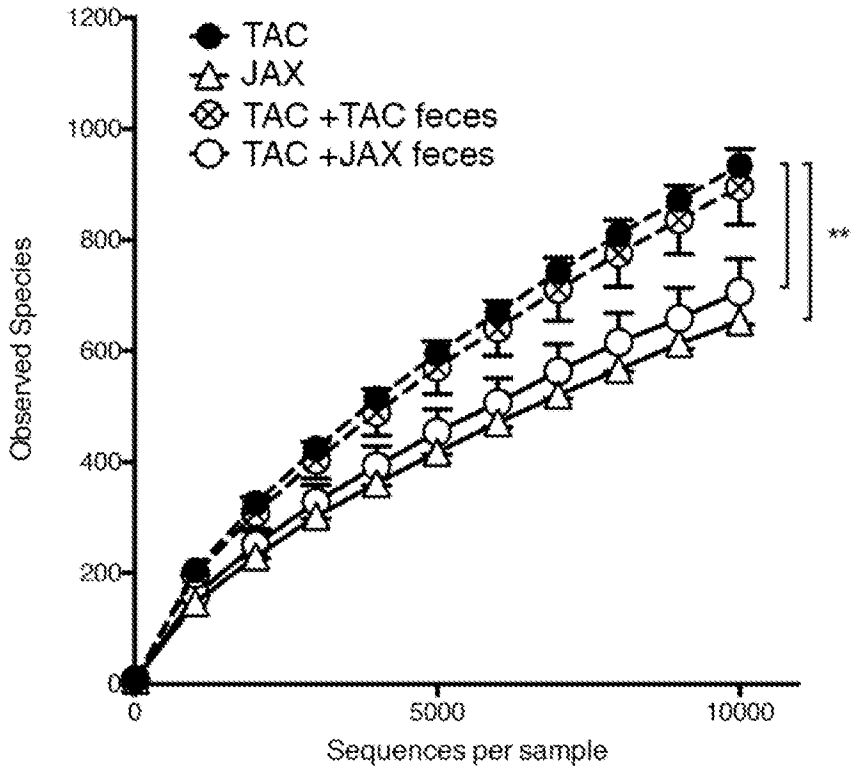

TAC
JAX
TAC +TAC feces
TAC +JAX feces
Observed Species
Sequences per sample
0
200
400
600
800
1000
1200
0
5000
10000
**

FIG. 3B

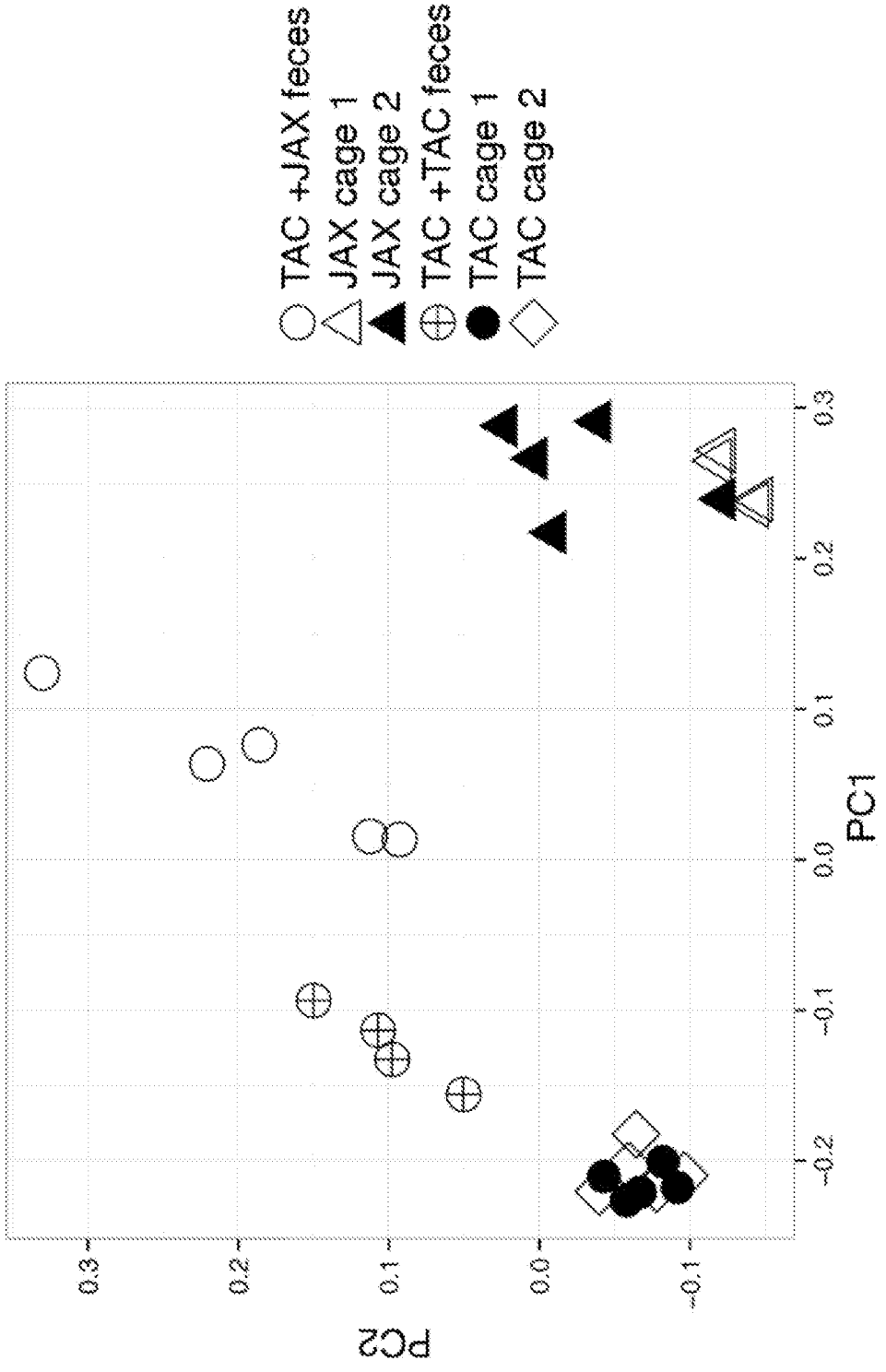

TAC +JAX feces
JAX cage 1
JAX cage 2
TAC +TAC feces
TAC cage 1
TAC cage 2
PC2
PC1
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.1
0.0
0.1
0.2
0.3

FIG. 3C

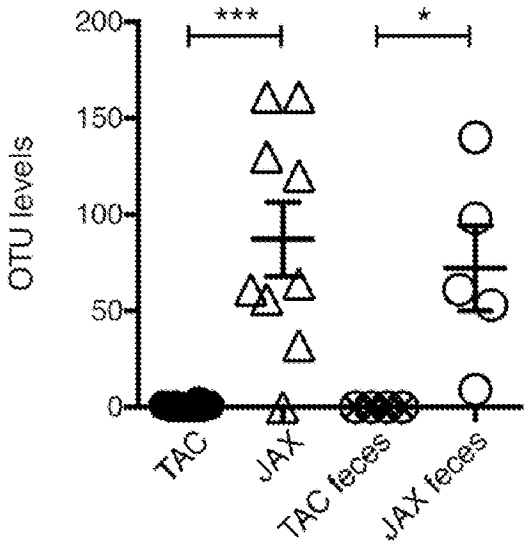


FIG. 3D

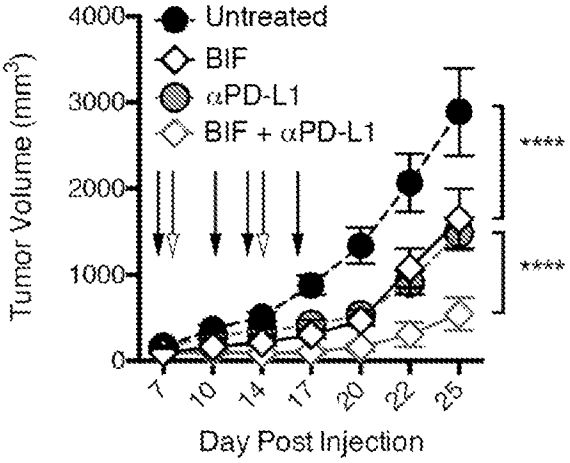


FIG. 3E

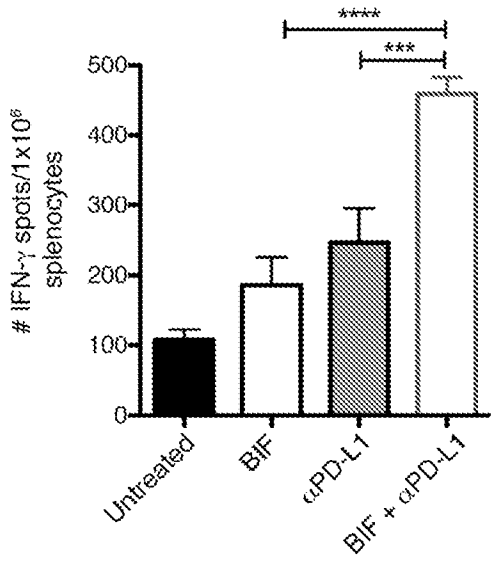


FIG. 3F

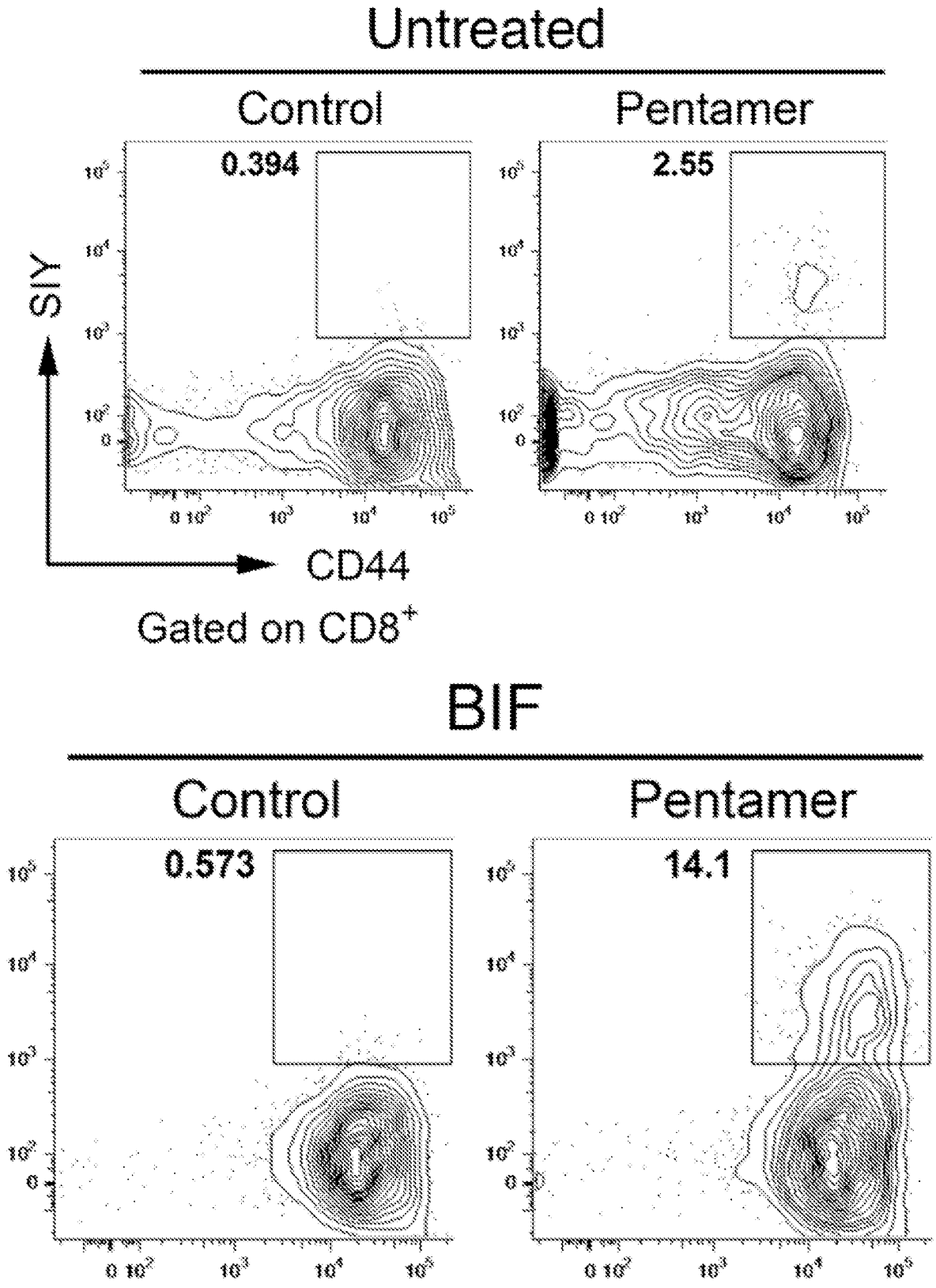

Untreated
Control
Pentamer
0.394
2.55
SIY
CD44
Gated on CD8+
BIF
Control
Pentamer
0.573
14.1

FIG. 3F (cont.)

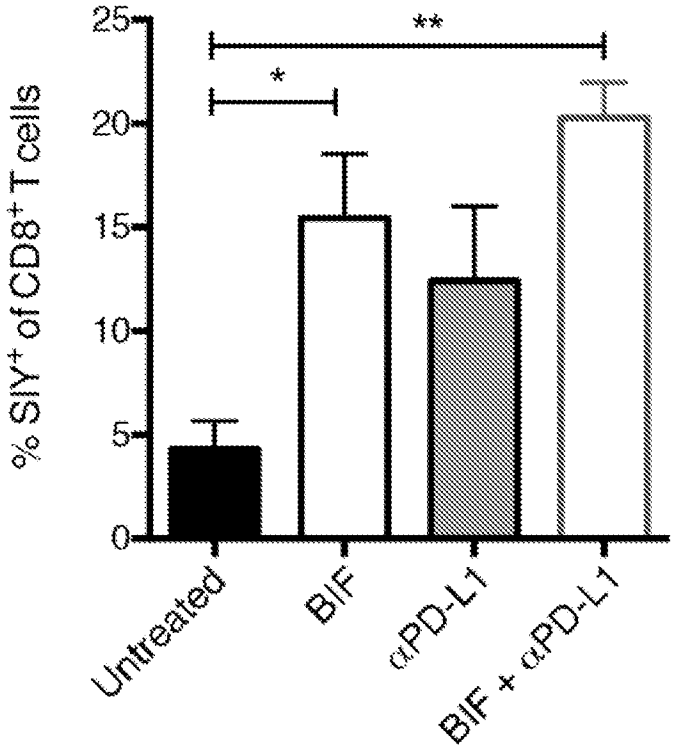


FIG. 3G

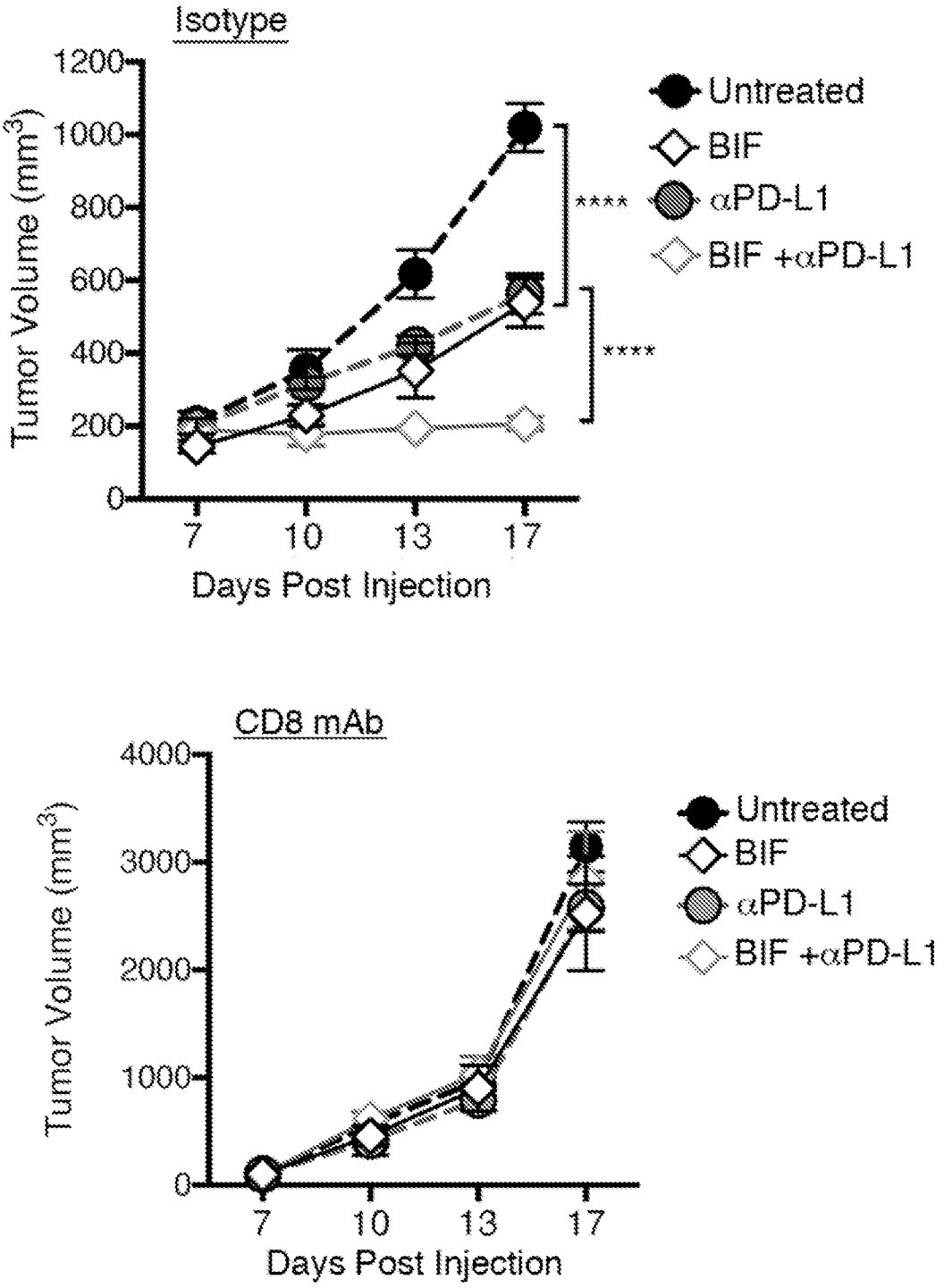

Isotype
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
7
10
13
17
Days Post Injection
Untreated
BIF
αPD-L1
BIF +αPD-L1
****
****
CD8 mAb
Tumor Volume (mm3)
4000
3000
2000
1000
0
7
10
13
17
Days Post Injection
Untreated
BIF
αPD-L1
BIF +αPD-L1

FIG. 4A

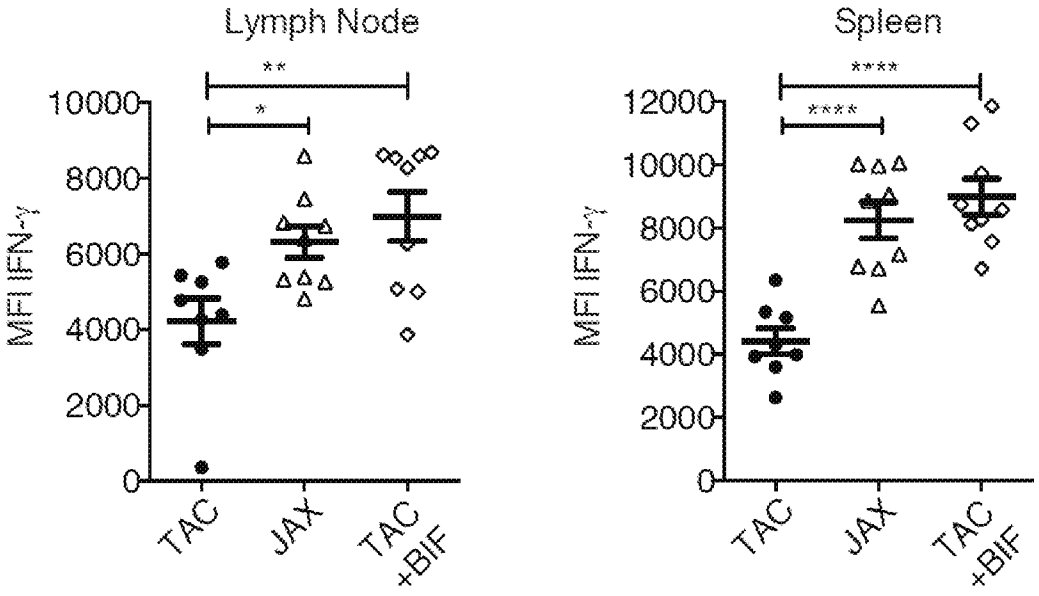

Lymph Node
Spleen
MFI IFN-γ
10000
8000
6000
4000
2000
0
12000
**
*
****
TAC
JAX
TAC +BIF

FIG. 4B

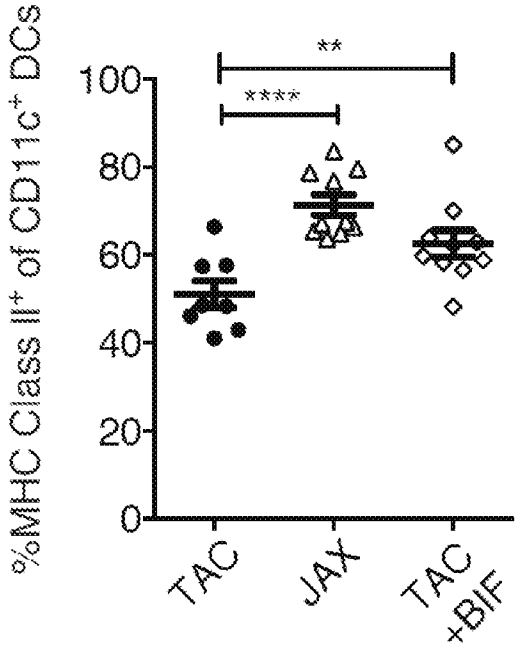

%MHC Class II+ of CD11c+ DCs
100
80
60
40
20
0
**
****
TAC
JAX
TAC +BIF

FIG. 4C

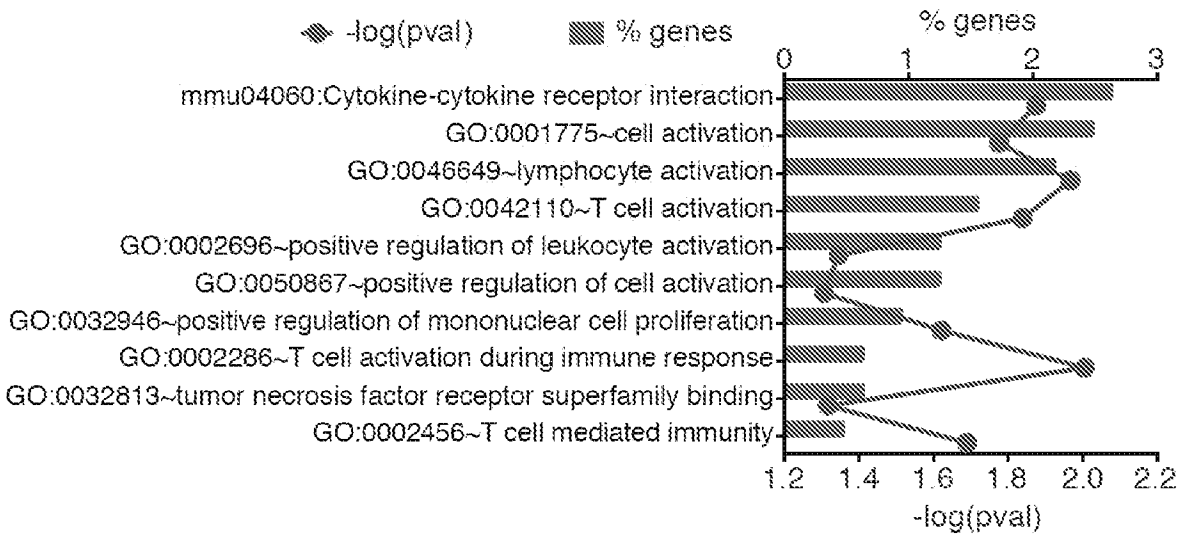

-log(pval)
% genes
% genes
0
1
2
3
mmu04060:Cytokine-cytokine receptor interaction
GO:0001775~cell activation
GO:0046649~lymphocyte activation
GO:0042110~T cell activation
GO:0002696~positive regulation of leukocyte activation
GO:0050867~positive regulation of cell activation
GO:0032946~positive regulation of mononuclear cell proliferation
GO:0002286~T cell activation during immune response
GO:0032813~tumor necrosis factor receptor superfamily binding
GO:0002456~T cell mediated immunity
1.2
1.4
1.6
1.8
2.0
2.2
-log(pval)

FIG. 4E

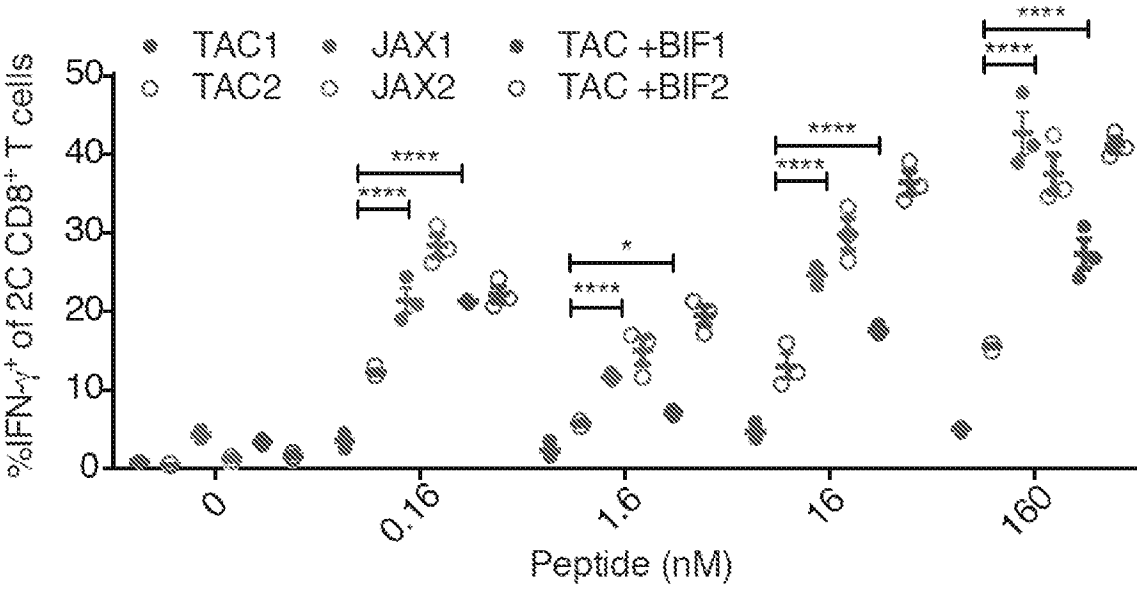
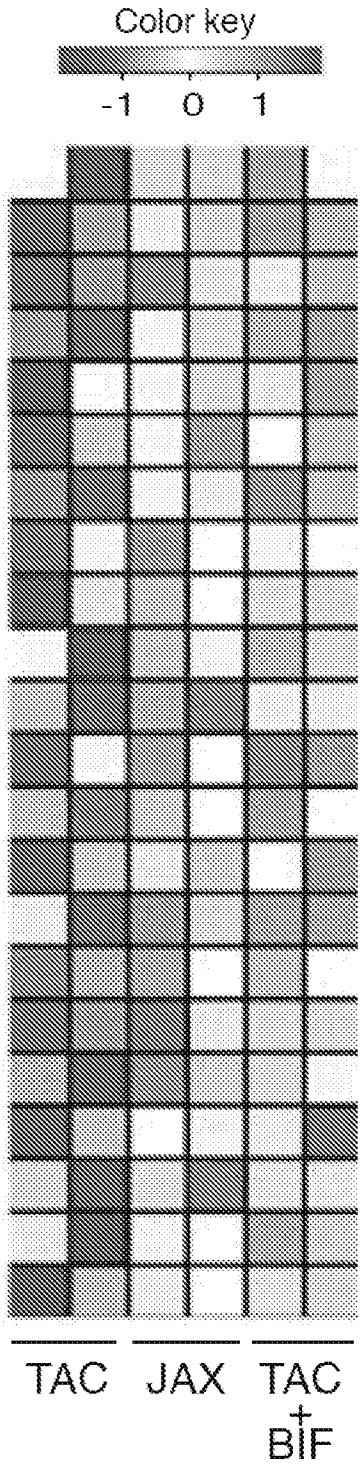

TAC1
TAC2
JAX1
JAX2
TAC +BIF1
TAC +BIF2
%IFN-γ+ of 2C CD8+ T cells
50
40
30
20
10
0
****
*
0
0.16
1.6
16
160
Peptide (nM)

FIG. 4D



Gene	JAX/TAC	BIF/TAC
H2m2	2.18	6.99
Cd40	1.58	2.11
Cd70	9.67	4.33
Icam1	1.57	2.00
Tnfrsf18	1.51	1.68
Tnfsf14	2.05	1.70
Relb	1.79	2.54
Ifngr2	3.10	1.71
Tapbp	42.40	14.97
Rab27a	2.55	2.96
Slc11a1	3.46	1.79
Cxcl9	2.64	1.53
Cx3cl1	3.96	6.36
Cxcr4	2.20	2.41
Irf1	1.55	2.44
Ifnar2	2.70	2.33
Oas2	1.68	1.51
Ifi35	1.92	1.57
Ifitm1	2.82	5.00
Azi2	7.71	3.04
Ikbke	1.60	2.03
Ulk1	5.78	3.51

FIG. 5A

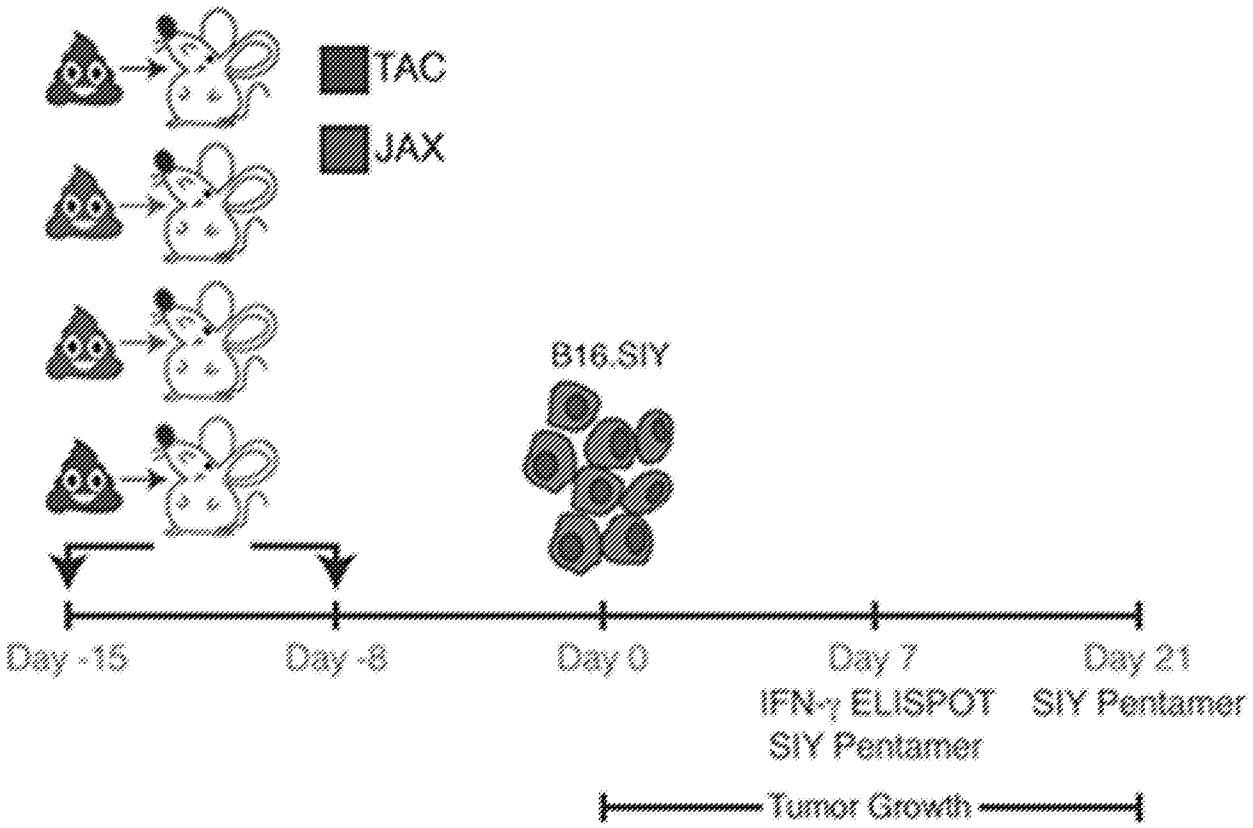


FIG. 5B

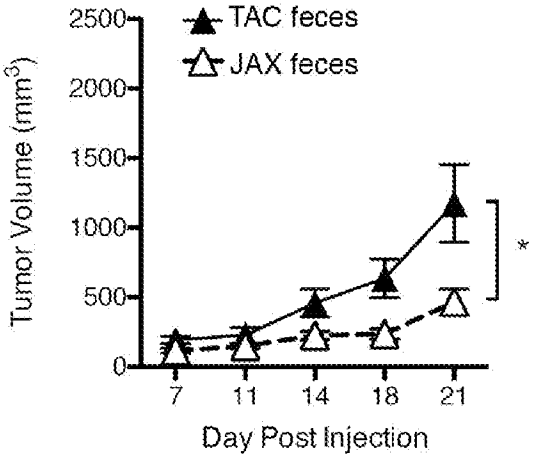


FIG. 5C

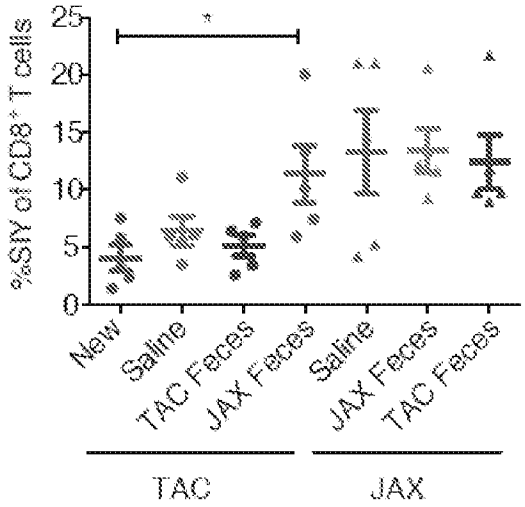


FIG. 5D

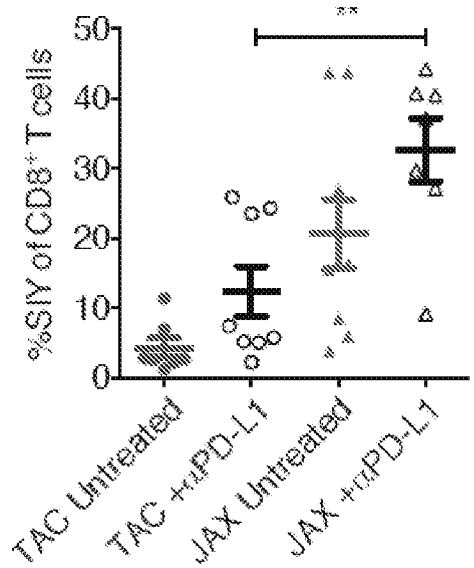


FIG. 6A

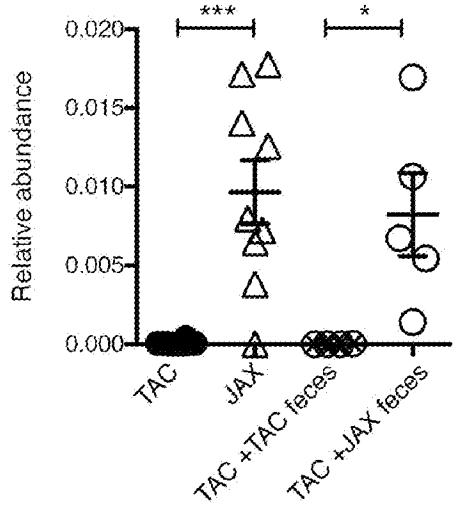


FIG. 6B

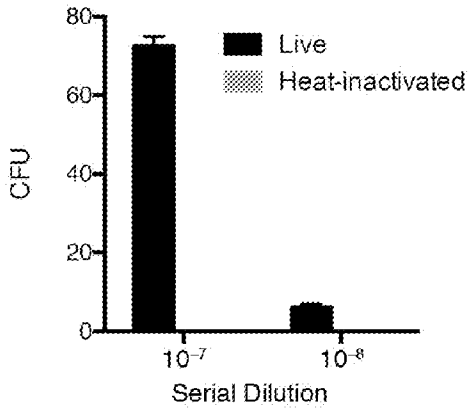


FIG. 6C

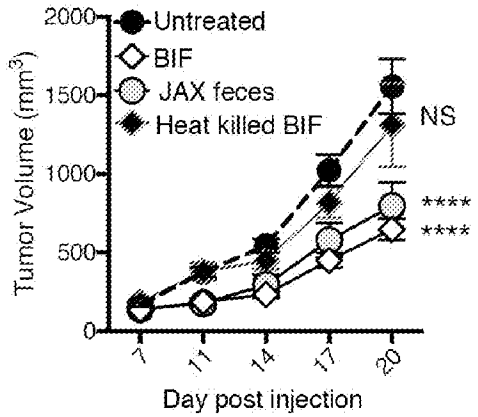


FIG. 6D

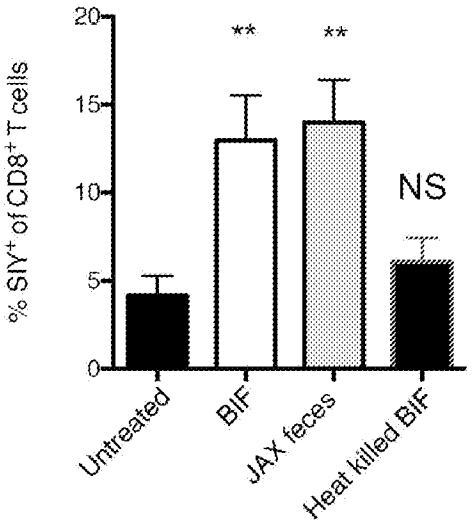


FIG. 6E

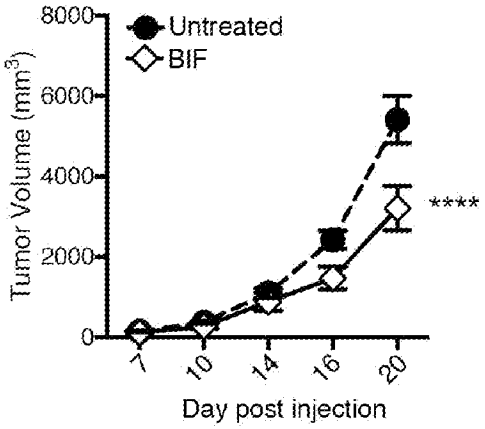


FIG. 6F

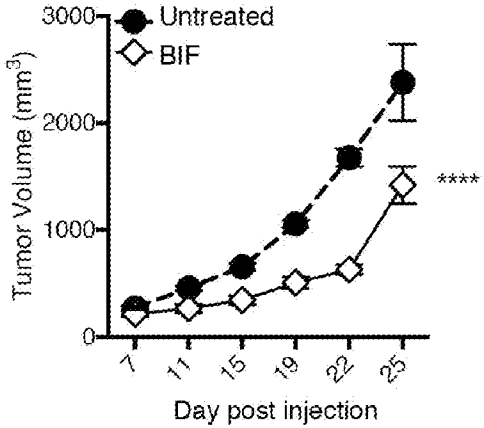


FIG. 6G

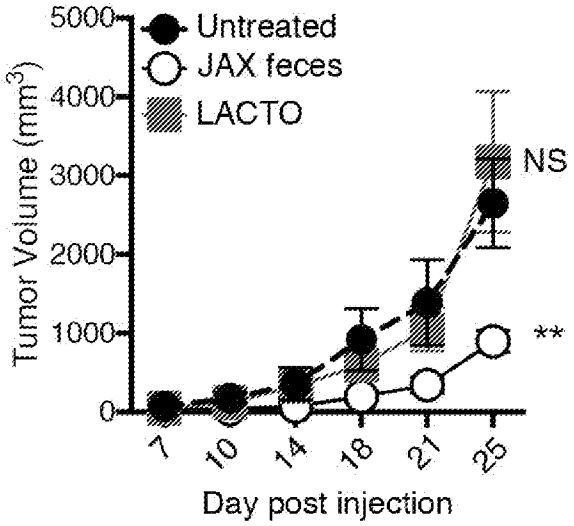


FIG. 6H

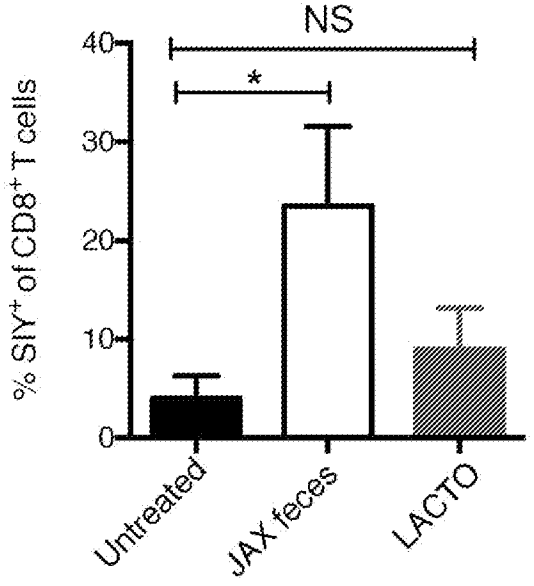


FIG. 7A

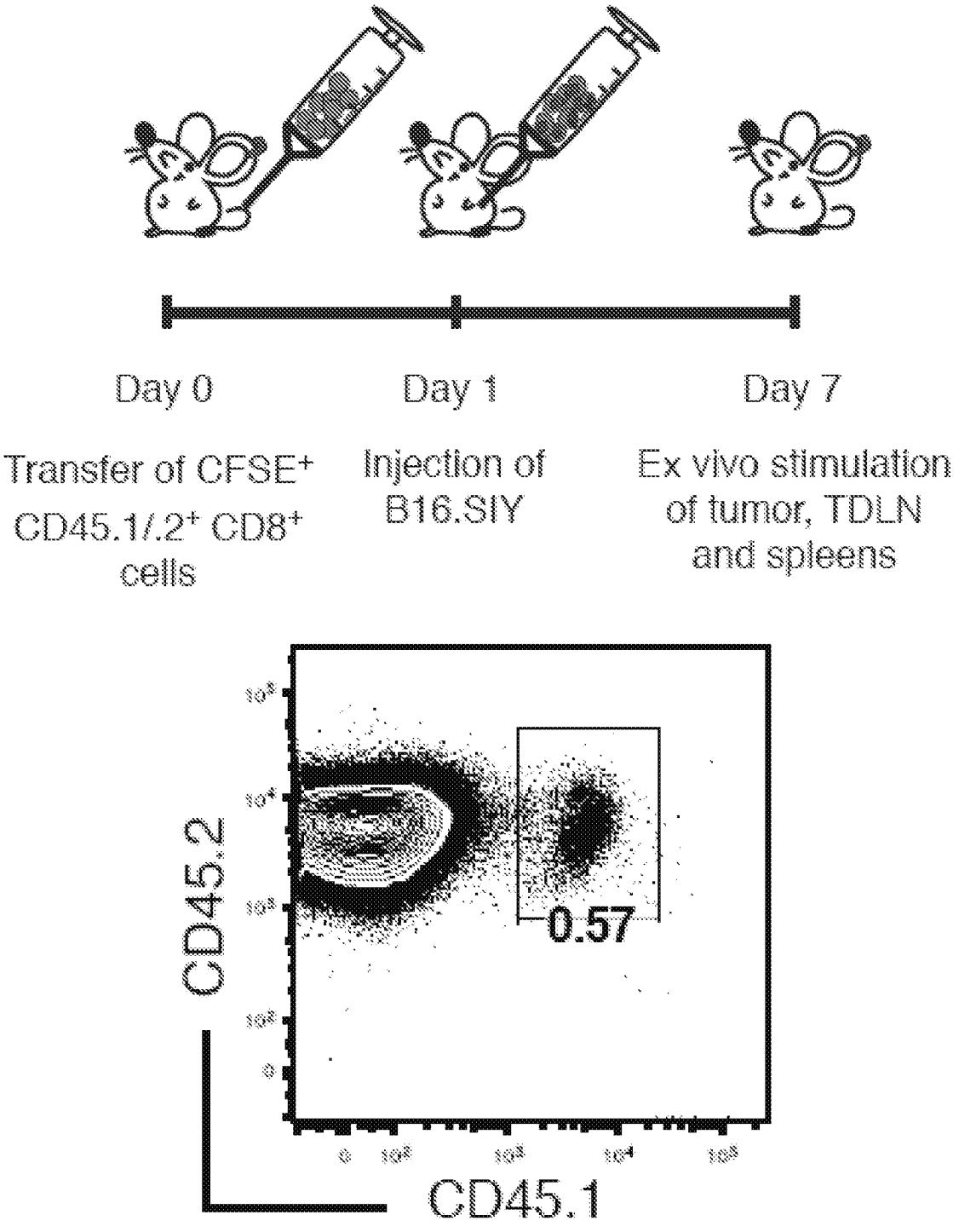

Day 0
Day 1
Day 7
Transfer of CFSE+ CD45.1/.2+ CD8+ cells
Injection of B16.SIY
Ex vivo stimulation of tumor, TDLN and spleens
CD45.2
CD45.1
0.57

FIG. 7B

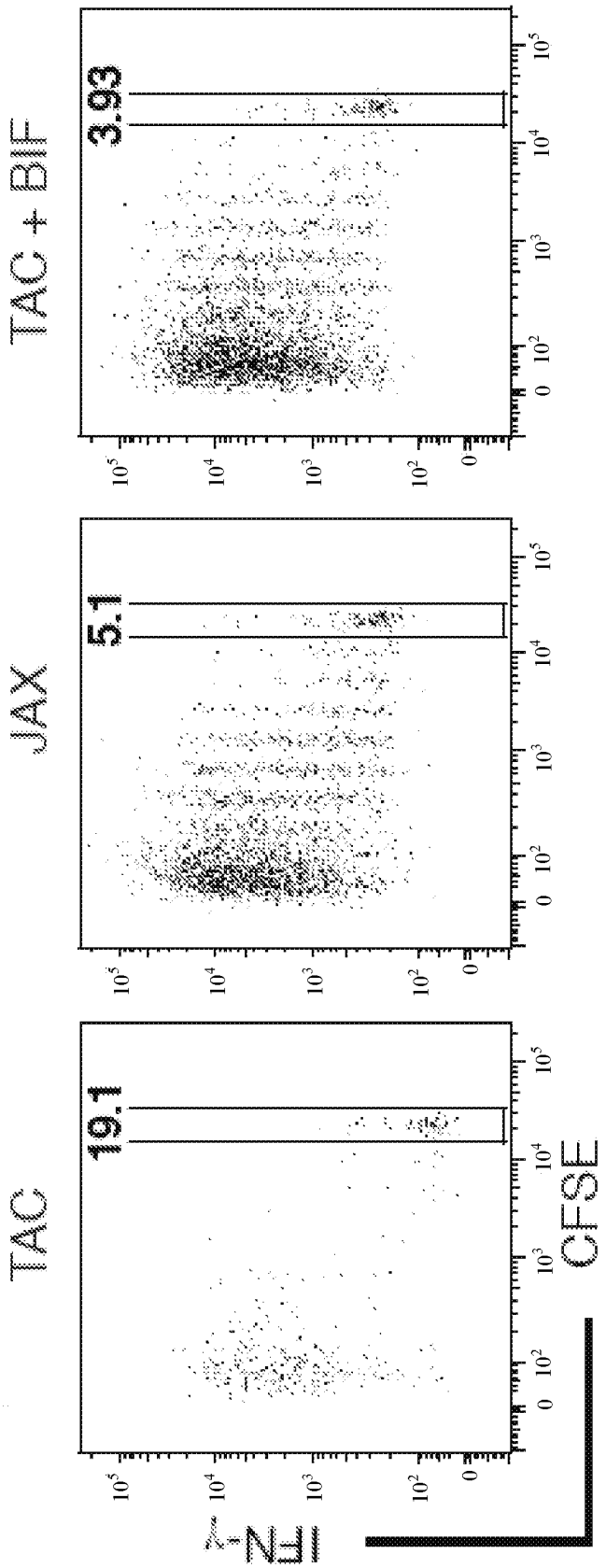


FIG. 7B (cont.)

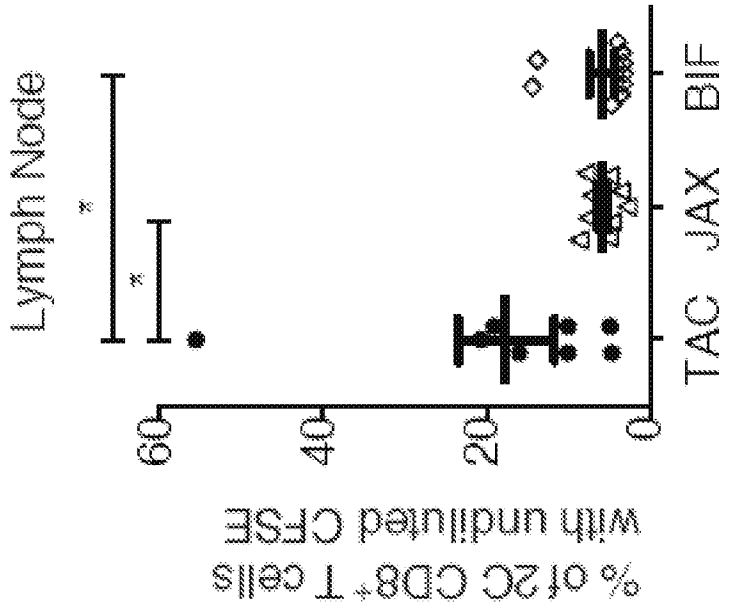

Lymph Node
% of 2C CD8+ T cells with undiluted CFSE
60
40
20
0
TAC
JAX
BIF
*
*

FIG. 8A

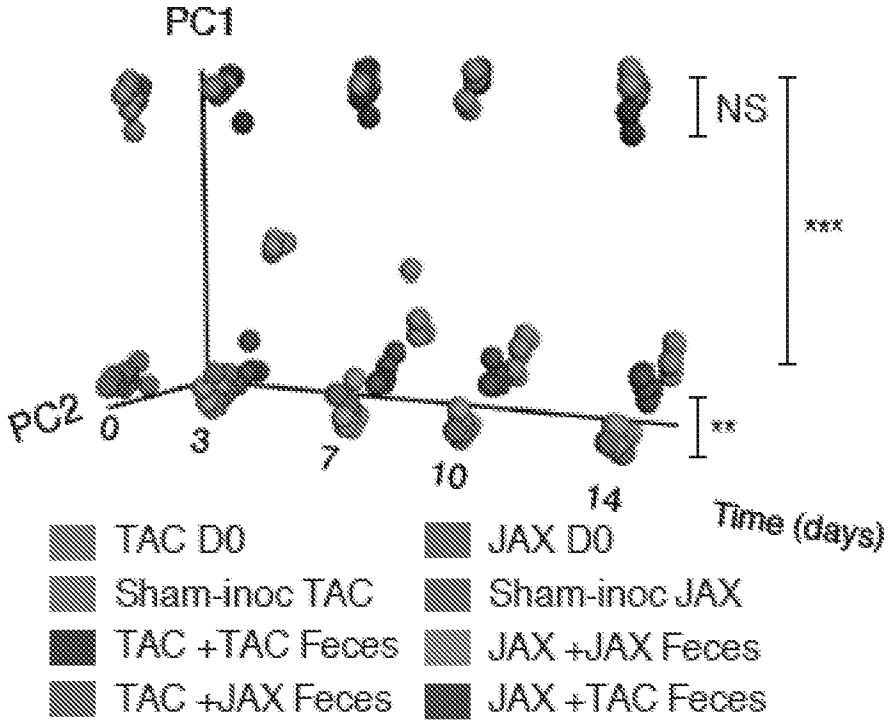
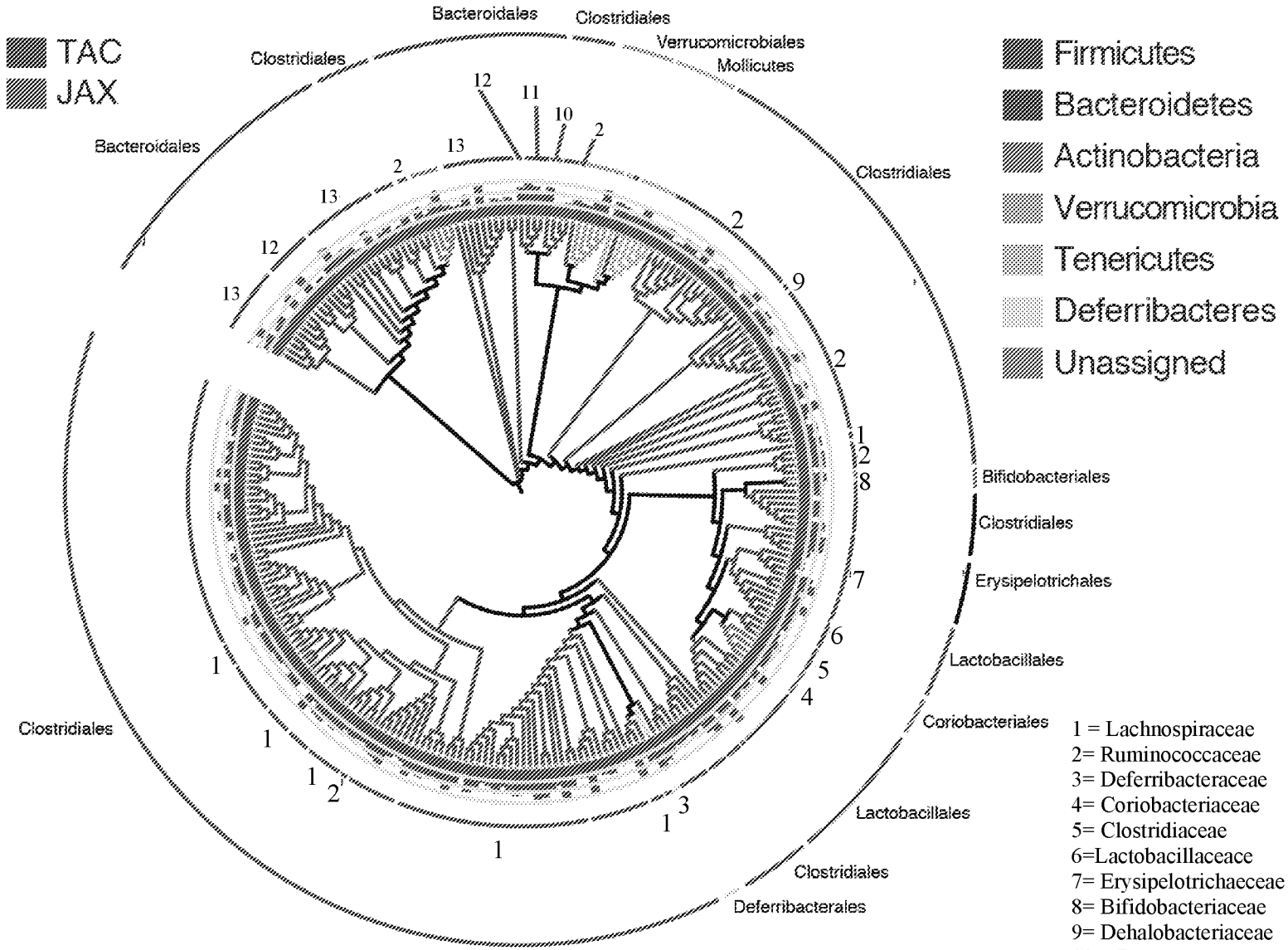

PC1
PC2
NS
***
**
0
3
7
10
14
Time (days)
TAC D0
Sham-inoc TAC
TAC +TAC Feces
TAC +JAX Feces
JAX D0
Sham-inoc JAX
JAX +JAX Feces
JAX +TAC Feces

FIG. 8B



1 = Lachnospiraceae
2= Ruminococcaceae
3= Deferribacteraceae
4= Coriobacteriaceae
5= Clostridiaceae
6=Lactobacillaceace
7= Erysipelotrichaeceae
8= Bifidobacteriaceae
9= Dehalobacteriaceae
10 = Peptostreptococceae
11= Rikenellaceae
12 = Porphyromonodaceae
13 = S24-7

FIG. 8C

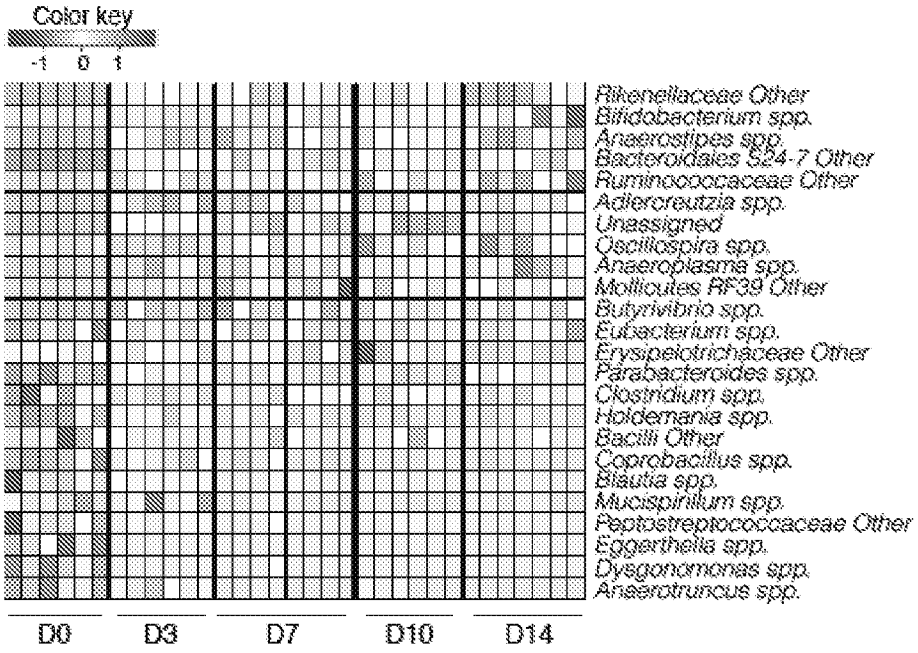

Color key
-1 0 1
Rikenellaceae Other
Bifidobacterium spp.
Anaerostipes spp.
Bacteroidales S24-7 Other
Ruminococcaceae Other
Adlercreutzia spp.
Unassigned
Oscillospira spp.
Anaeroplasma spp.
Mollicutes RF39 Other
Butyrivibrio spp.
Eubacterium spp.
Erysipelotrichaceae Other
Parabacteroides spp.
Clostridium spp.
Holdemania spp.
Bacilli Other
Coprobacillus spp.
Blautia spp.
Mucispirillum spp.
Peptostreptococcaceae Other
Eggerthella spp.
Dysgonomonas spp.
Anaerotruncus spp.
D0
D3
D7
D10
D14

FIG. 8D

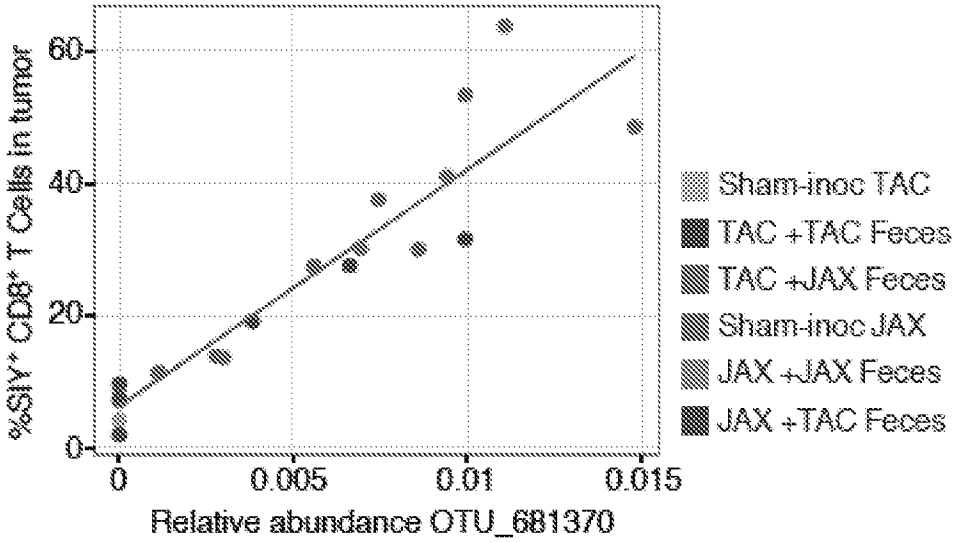

%SIY+ CD8+ T Cells in tumor
60
40
20
0
0
0.005
0.01
0.015
Relative abundance OTU_681370
Sham-inoc TAC
TAC +TAC Feces
TAC +JAX Feces
Sham-inoc JAX
JAX +JAX Feces
JAX +TAC Feces

FIG. 8E

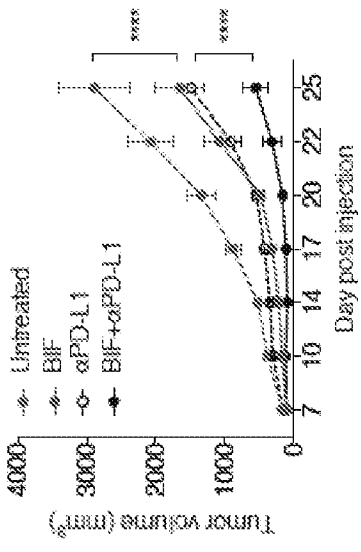


FIG. 8F

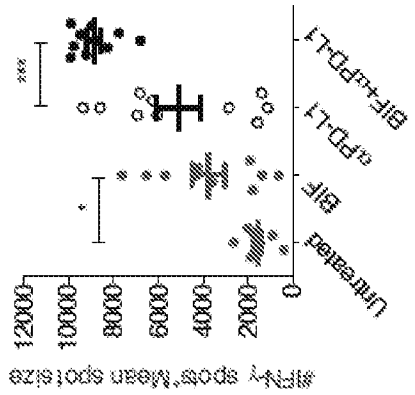


FIG. 8G

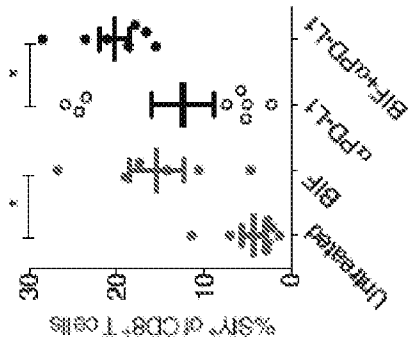


FIG. 9A

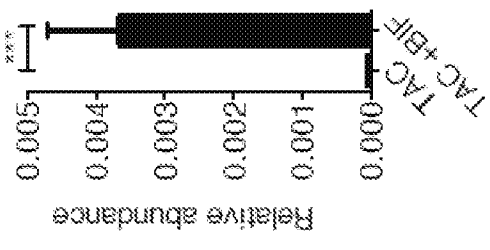


FIG. 9B

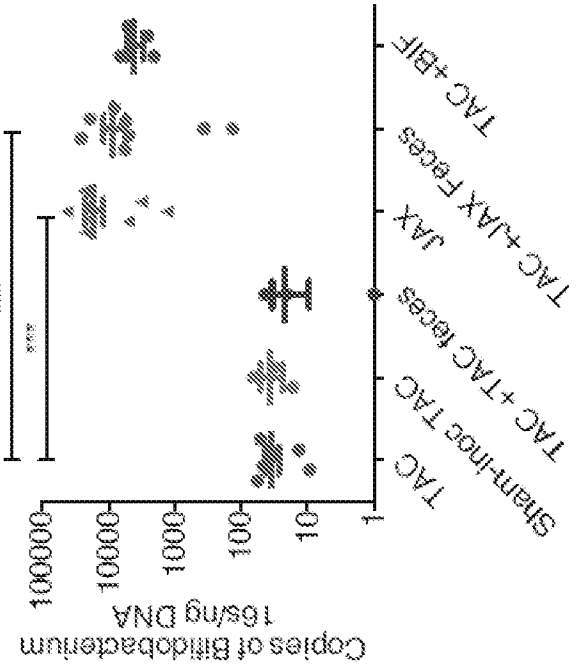


FIG. 9D

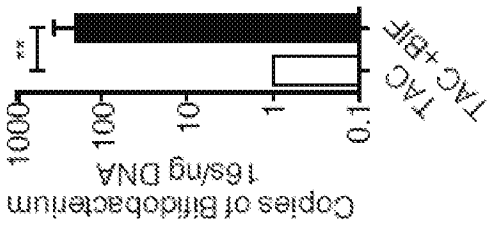


FIG. 9C

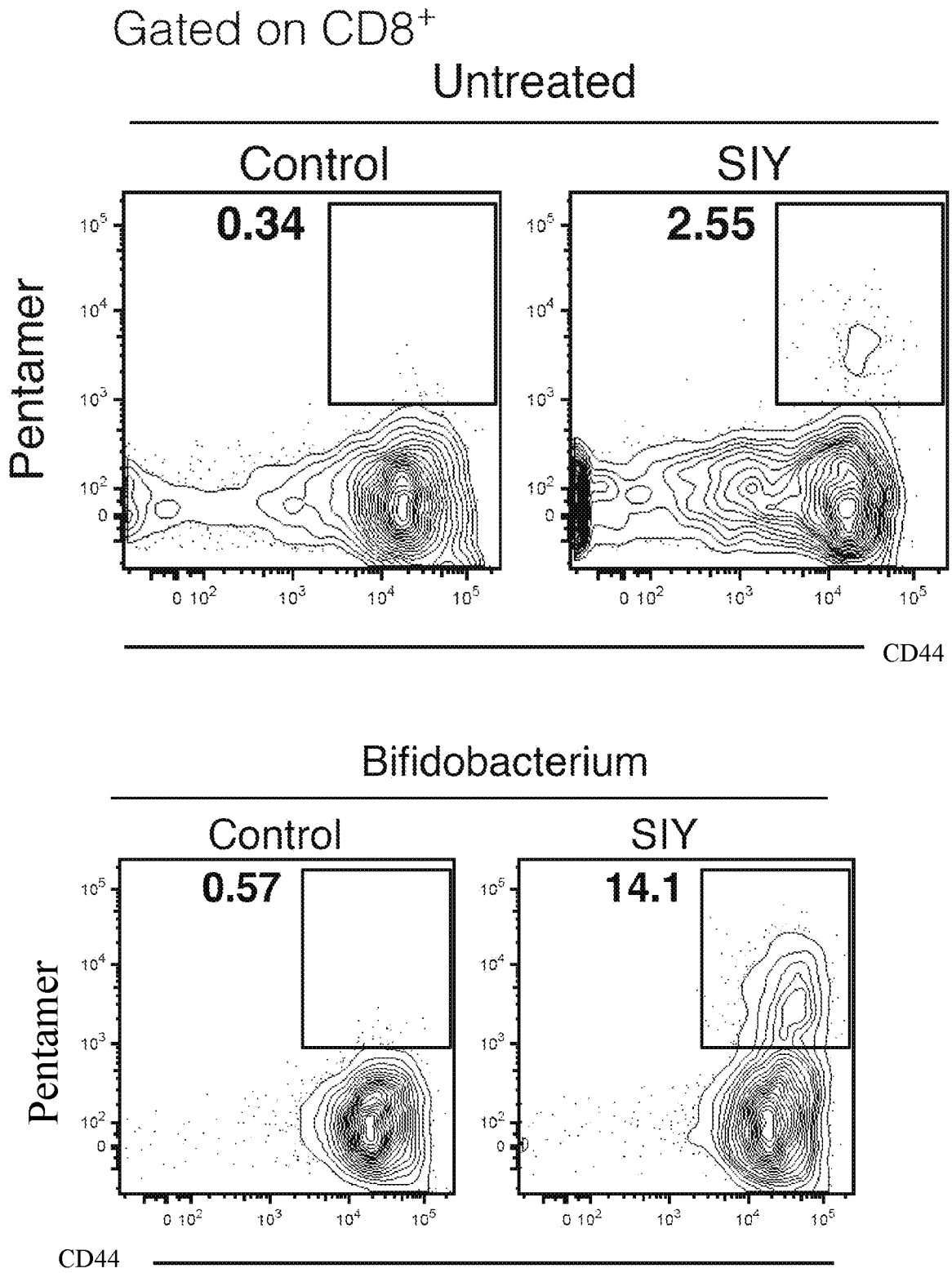

Gated on CD8+
Untreated
Control
SIY
0.34
2.55
Pentamer
CD44
Bifidobacterium
Control
SIY
0.57
14.1
Pentamer
CD44

FIG. 9E

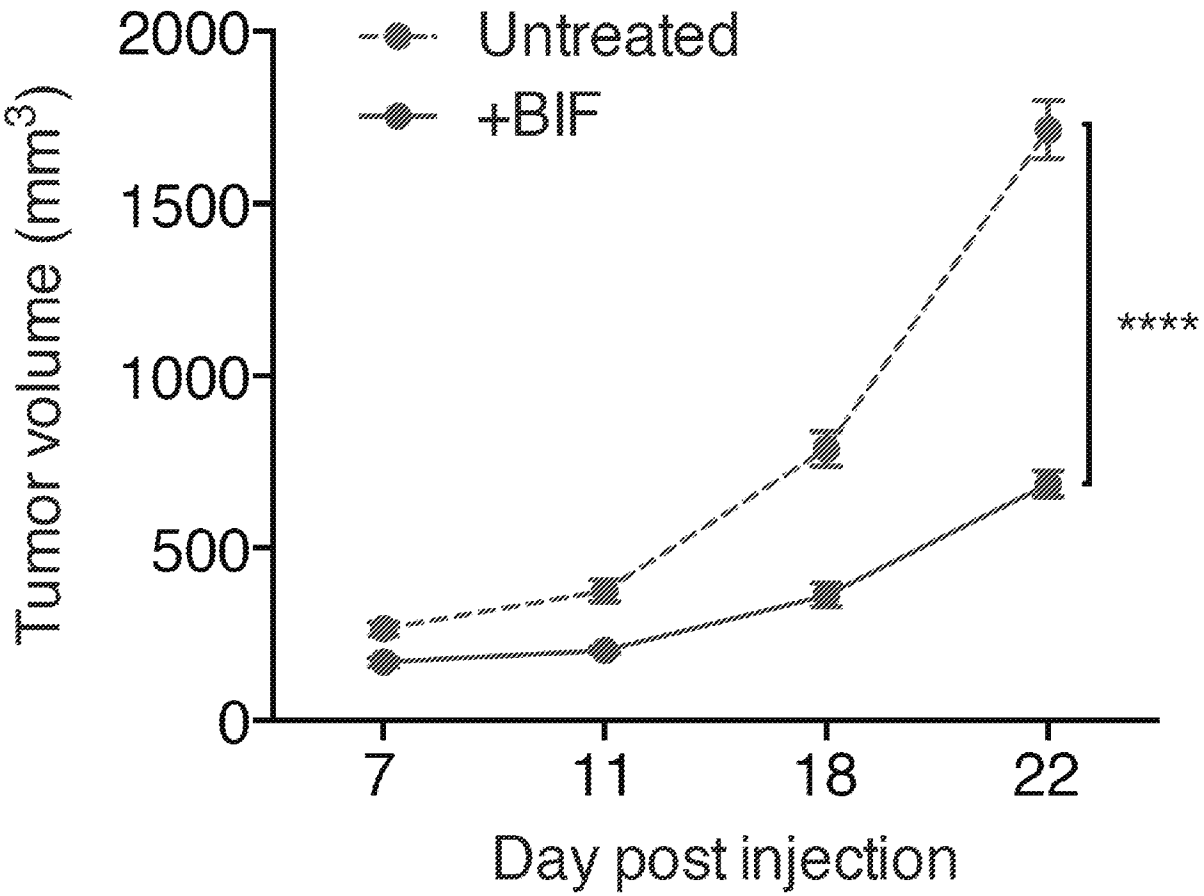


FIG. 10A

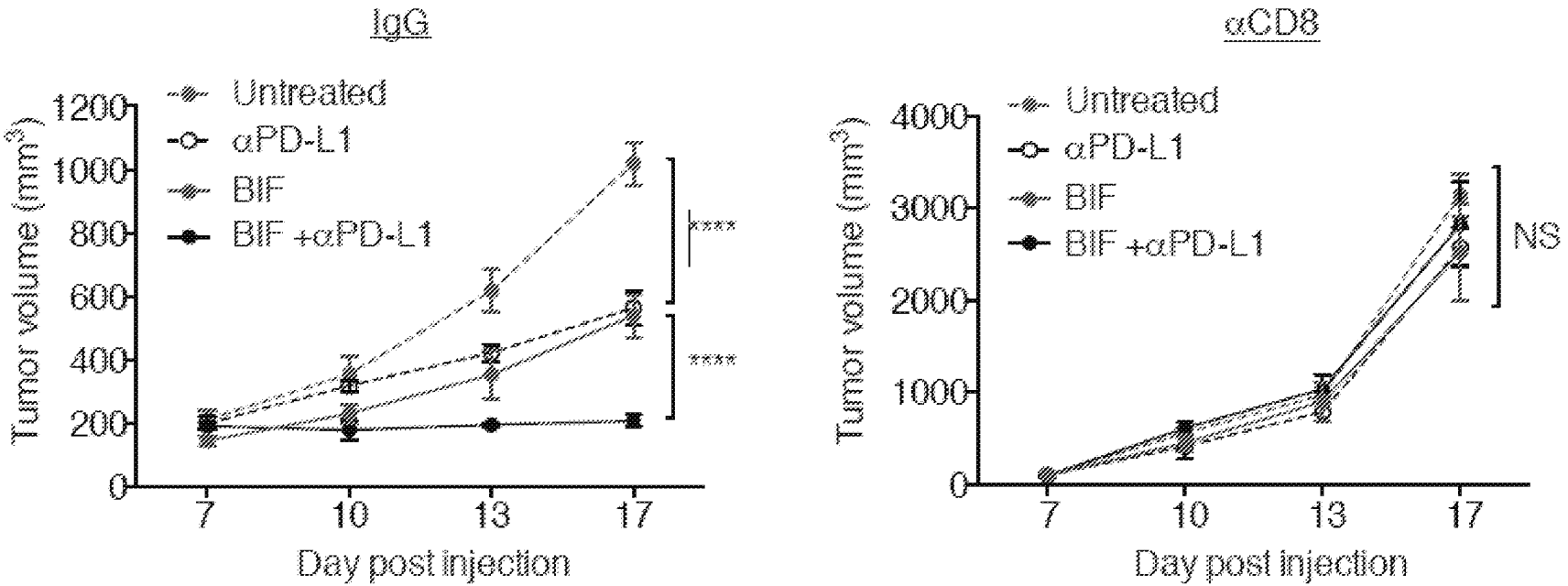


FIG. 10B

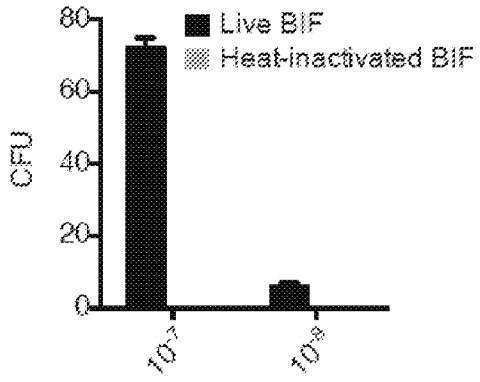


FIG. 10C

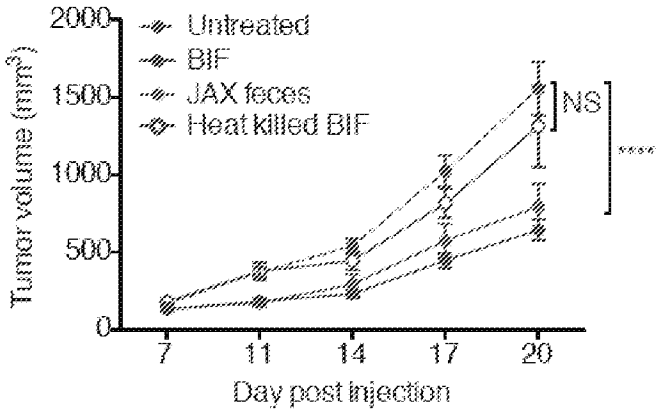


FIG. 10D

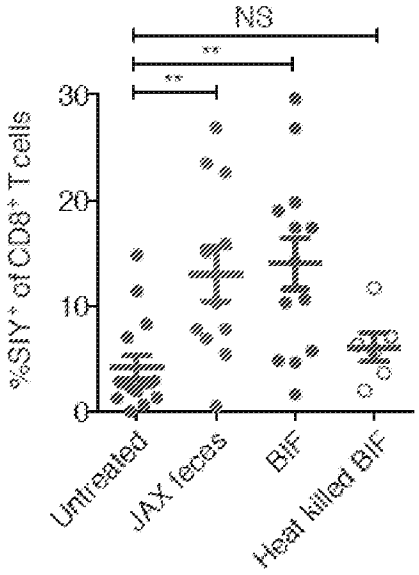


FIG. 11A

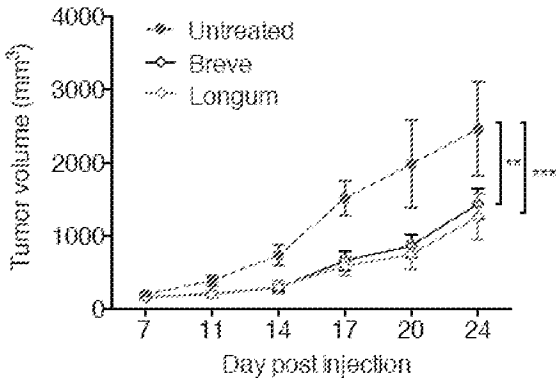


FIG. 11B

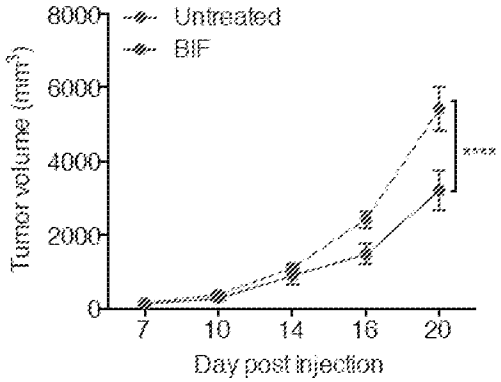


FIG. 11C

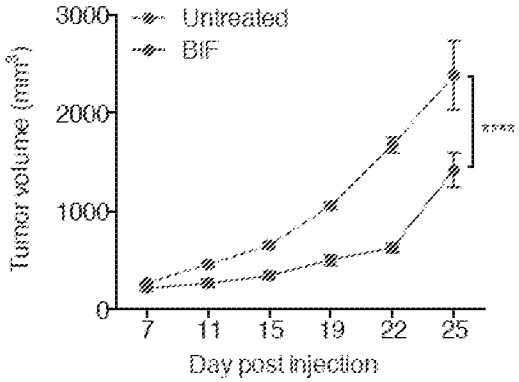


FIG. 11D

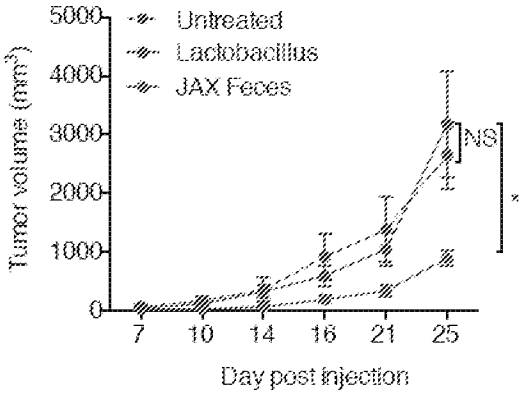
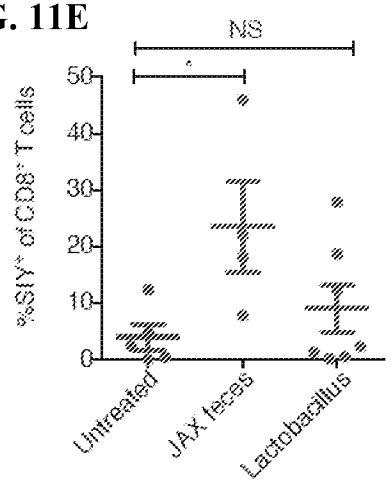


FIG. 11E



REPLACEMENT SHEET

FIG. 12A

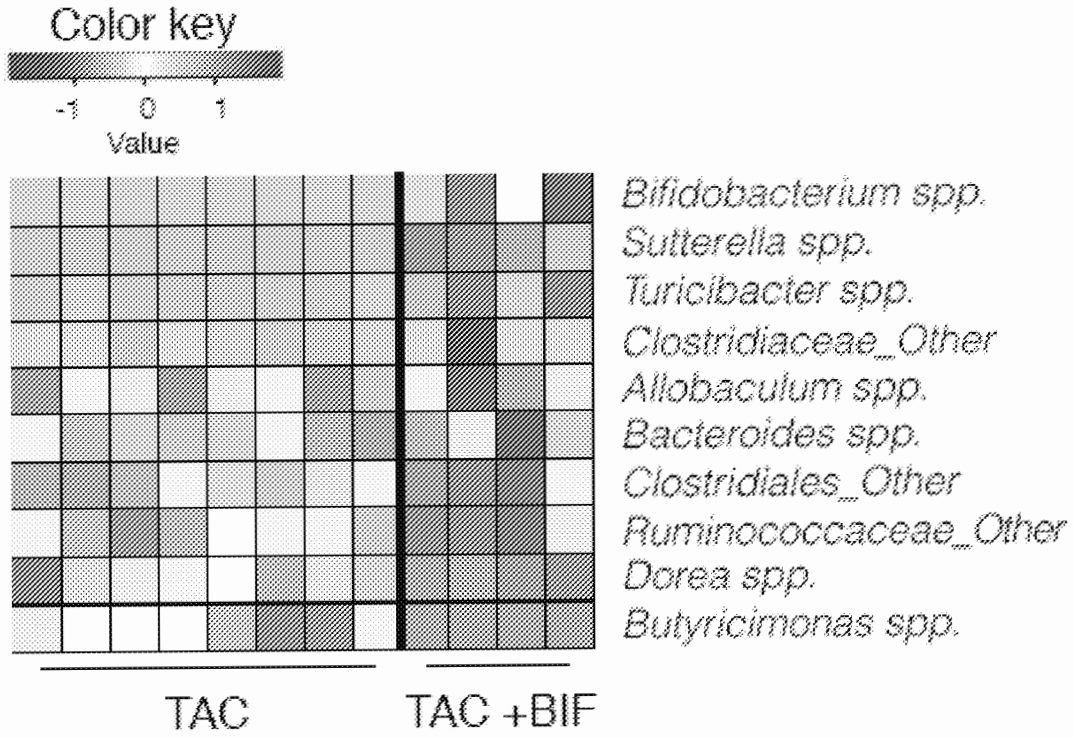
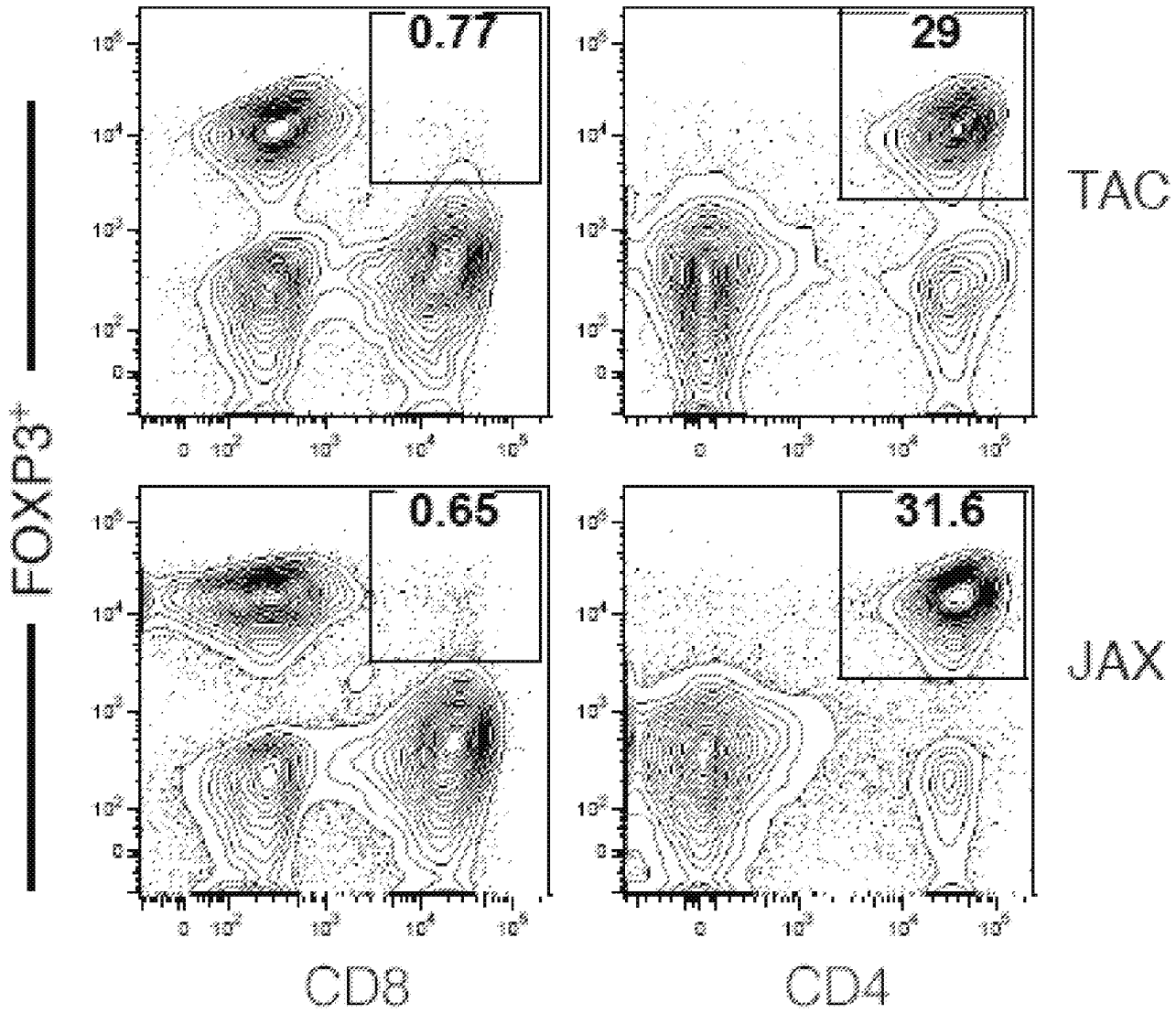
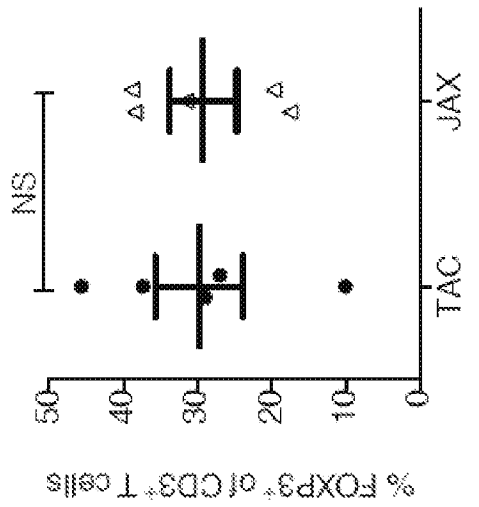


FIG. 12B




% FOXP3+ of CD3+ T cells
50
40
30
20
10
0
NS
TAC
JAX

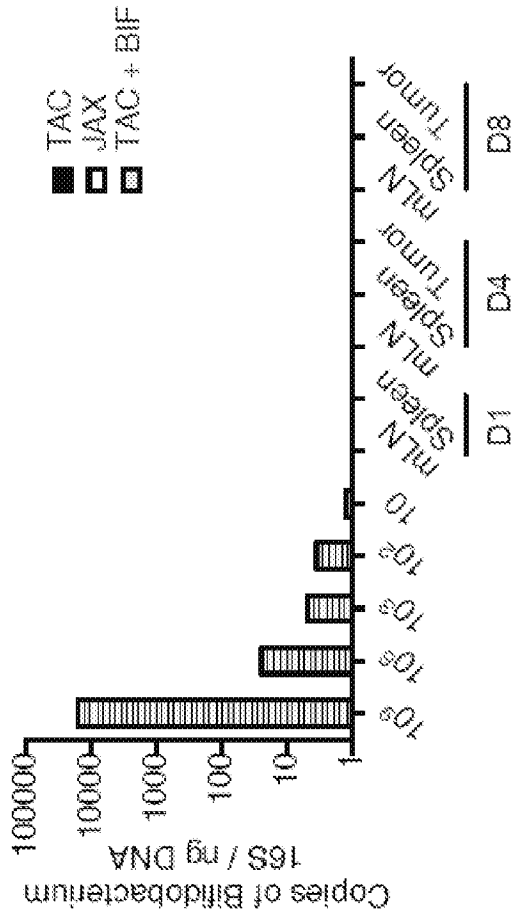

Copies of Bifidobacterium
16S / ng DNA
100000
10000
1000
100
10
1
TAC
JAX
TAC + BIF
10⁸
10⁷
10⁶
10⁵
10⁴
10
mLN
Spleen
mLN
Spleen
mLN
Spleen
Tumor
Tumor
D1
D4
D8

FIG. 12C

FIG. 13A

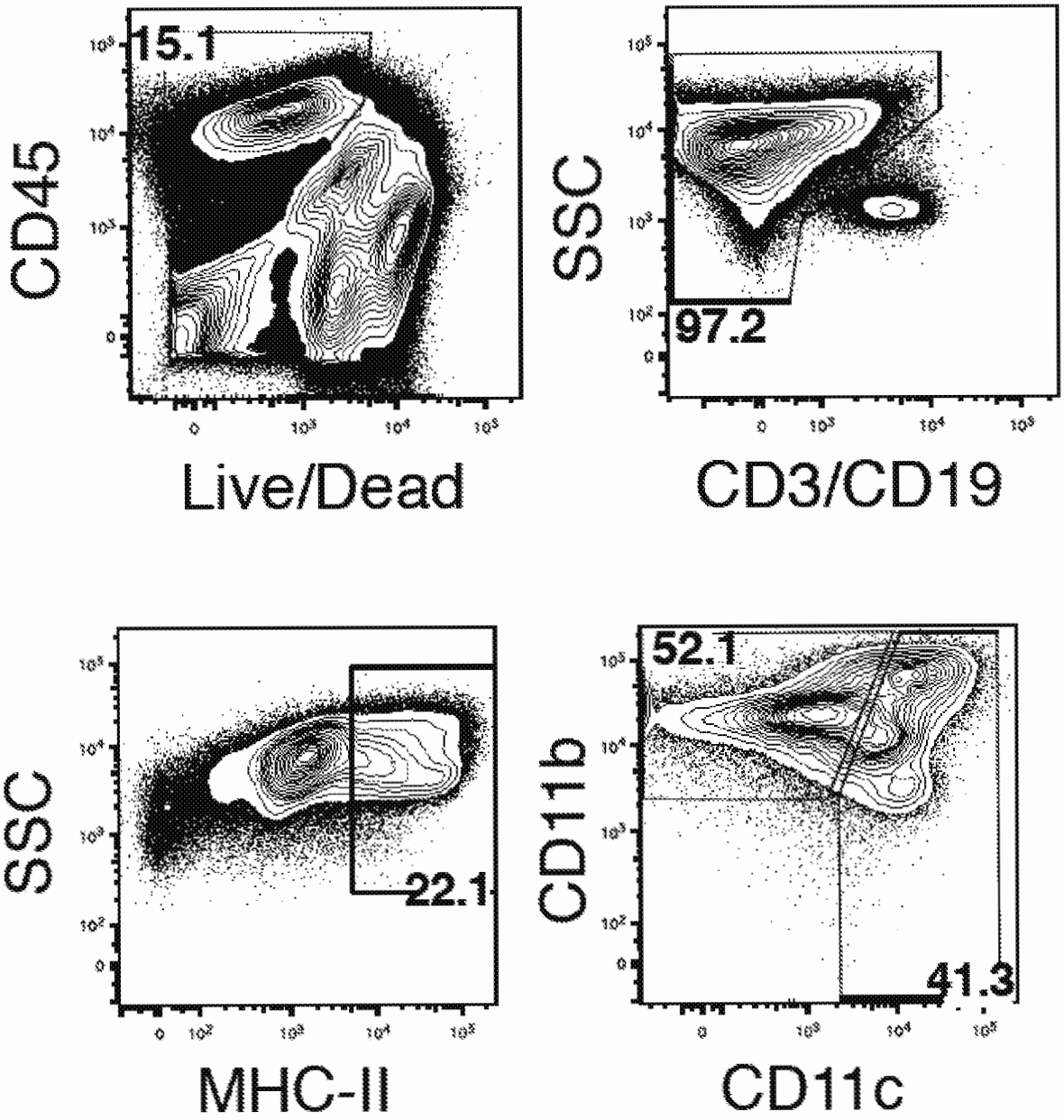

15.1
CD45
Live/Dead
SSC
97.2
CD3/CD19
SSC
22.1
MHC-II
52.1
CD11b
41.3
CD11c

FIG. 13B

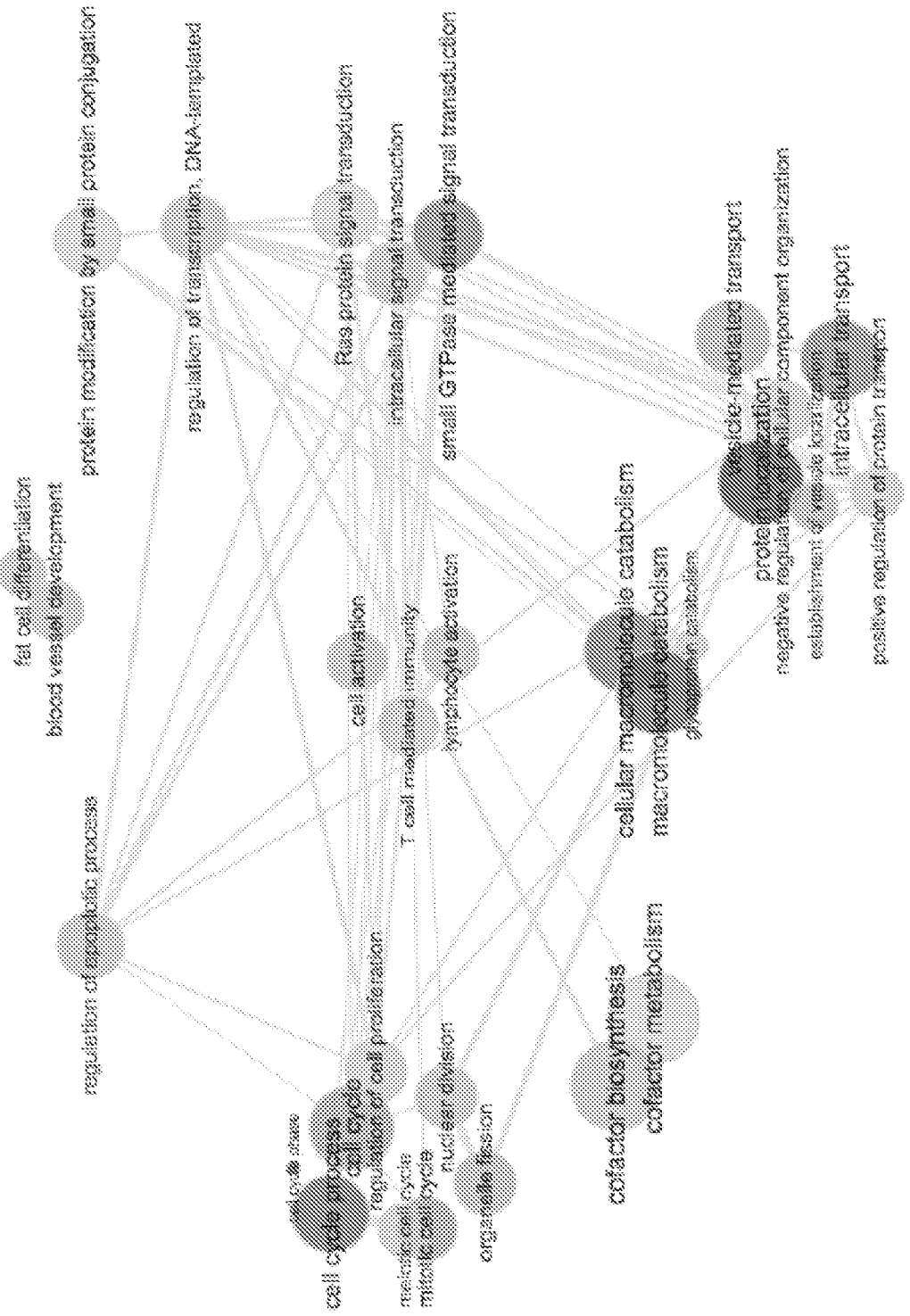

fat cell differentiation
blood vessel development
protein modification by small protein conjugation
regulation of apoptotic process
regulation of transcription, DNA-templated
Ras protein signal transduction
intracellular signal transduction
small GTPase mediated signal transduction
cell activation
T cell mediated immunity
lymphocyte activation
cellular macromolecule catabolism
macromolecule catabolism
cofactor biosynthesis
cofactor metabolism
negative regulation of cellular component organization
establishment of protein localization
intracellular transport
positive regulation of protein transport
cell cycle phase
cell cycle process
regulation of cell proliferation
meiotic cell cycle
mitotic cell cycle
nuclear division
organelle fission

FIG. 13C

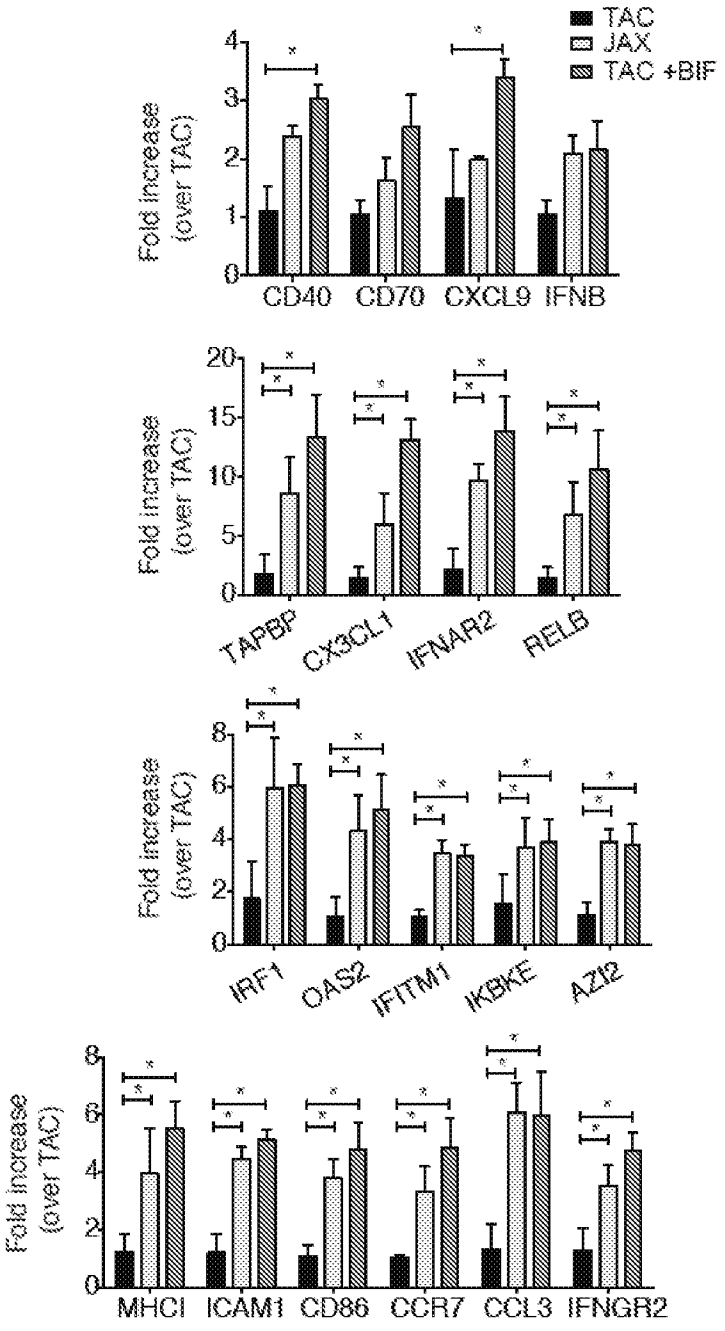

TAC
JAX
TAC +BIF
Fold increase (over TAC)
CD40
CD70
CXCL9
IFNB
TAPBP
CX3CL1
IFNAR2
RELB
IRF1
OAS2
IFITM1
IKBKE
AZI2
MHCI
ICAM1
CD86
CCR7
CCL3
IFNGR2

FIG. 14A

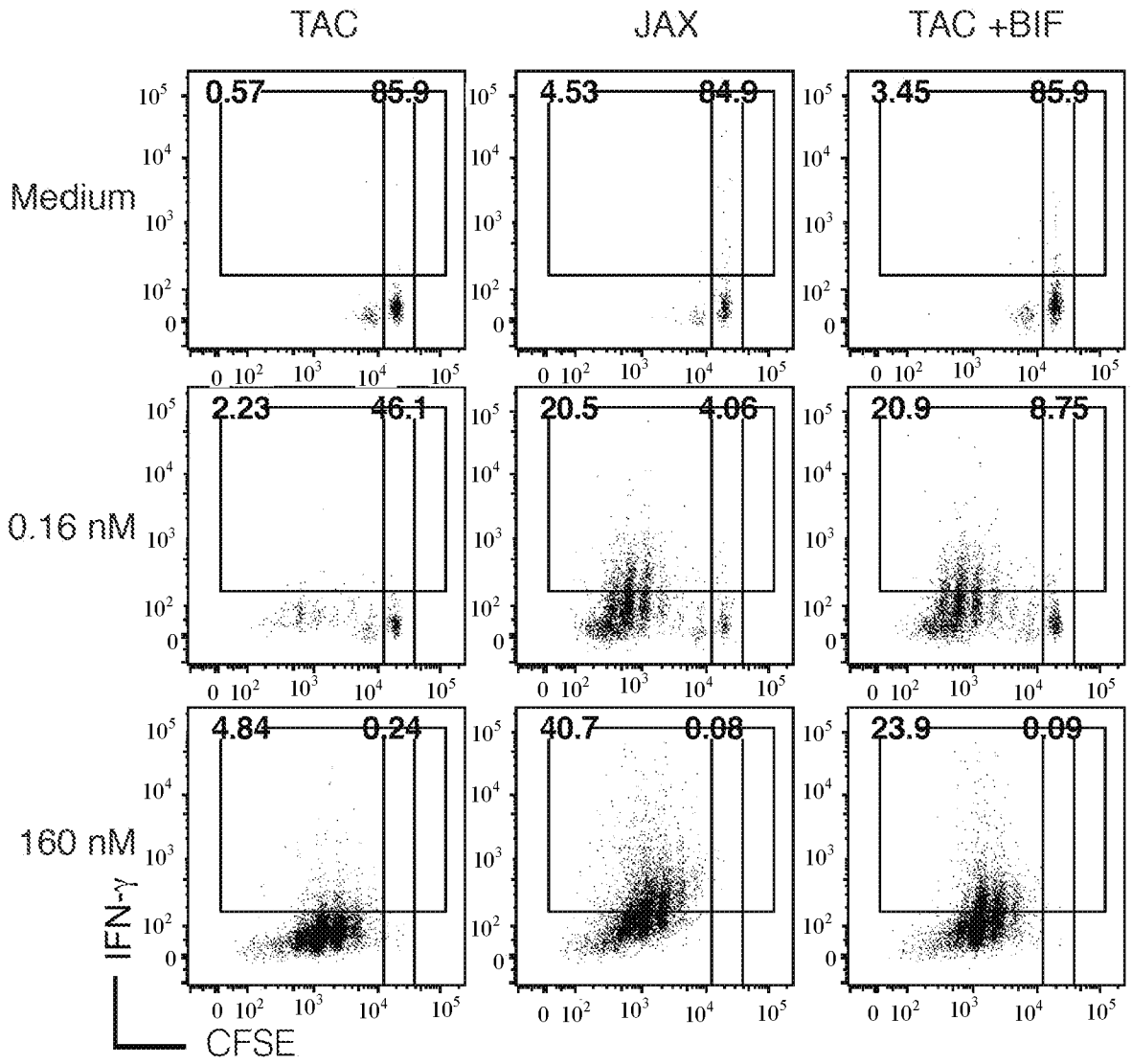

TAC
JAX
TAC +BIF
Medium
0.16 nM
160 nM
IFN-γ
CFSE
0.57
85.9
4.53
84.9
3.45
85.9
2.23
46.1
20.5
4.06
20.9
8.75
4.84
0.24
40.7
0.08
23.9
0.09

FIG. 14B

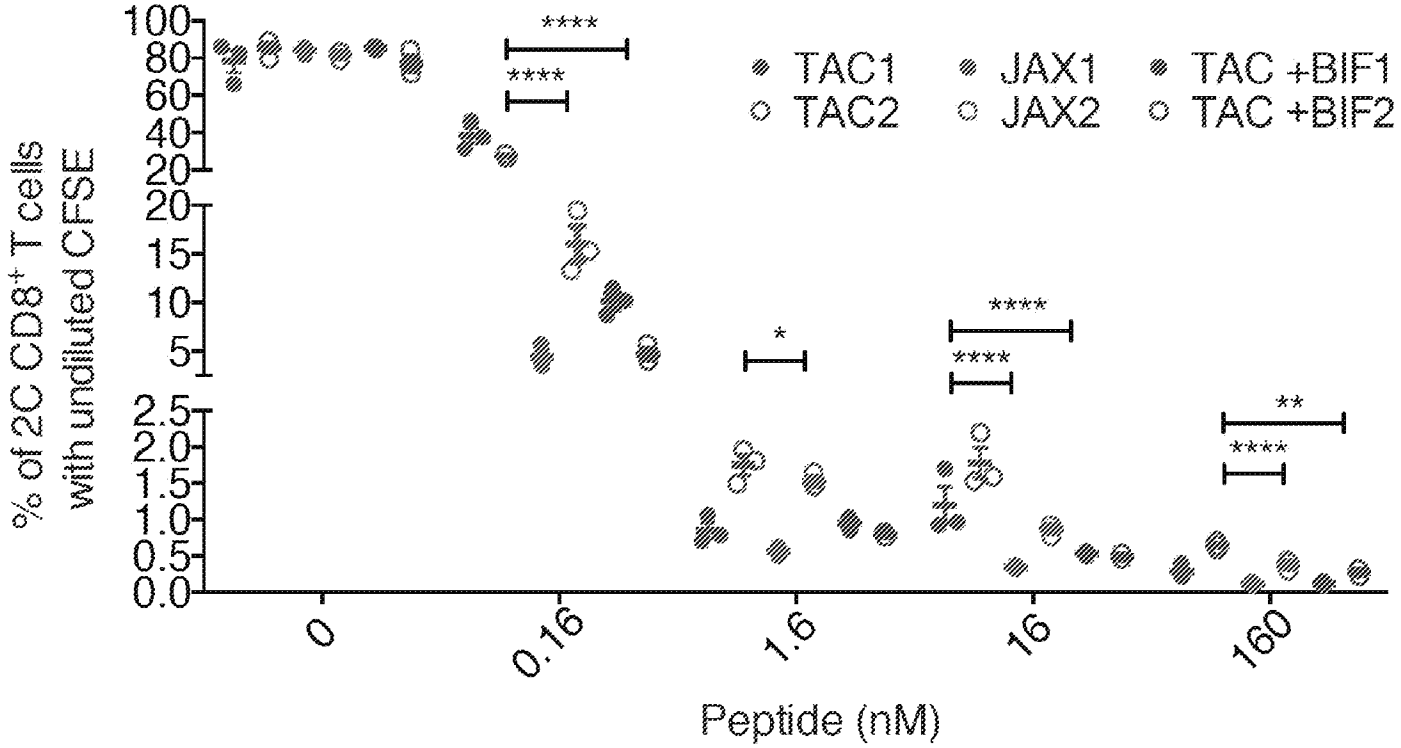
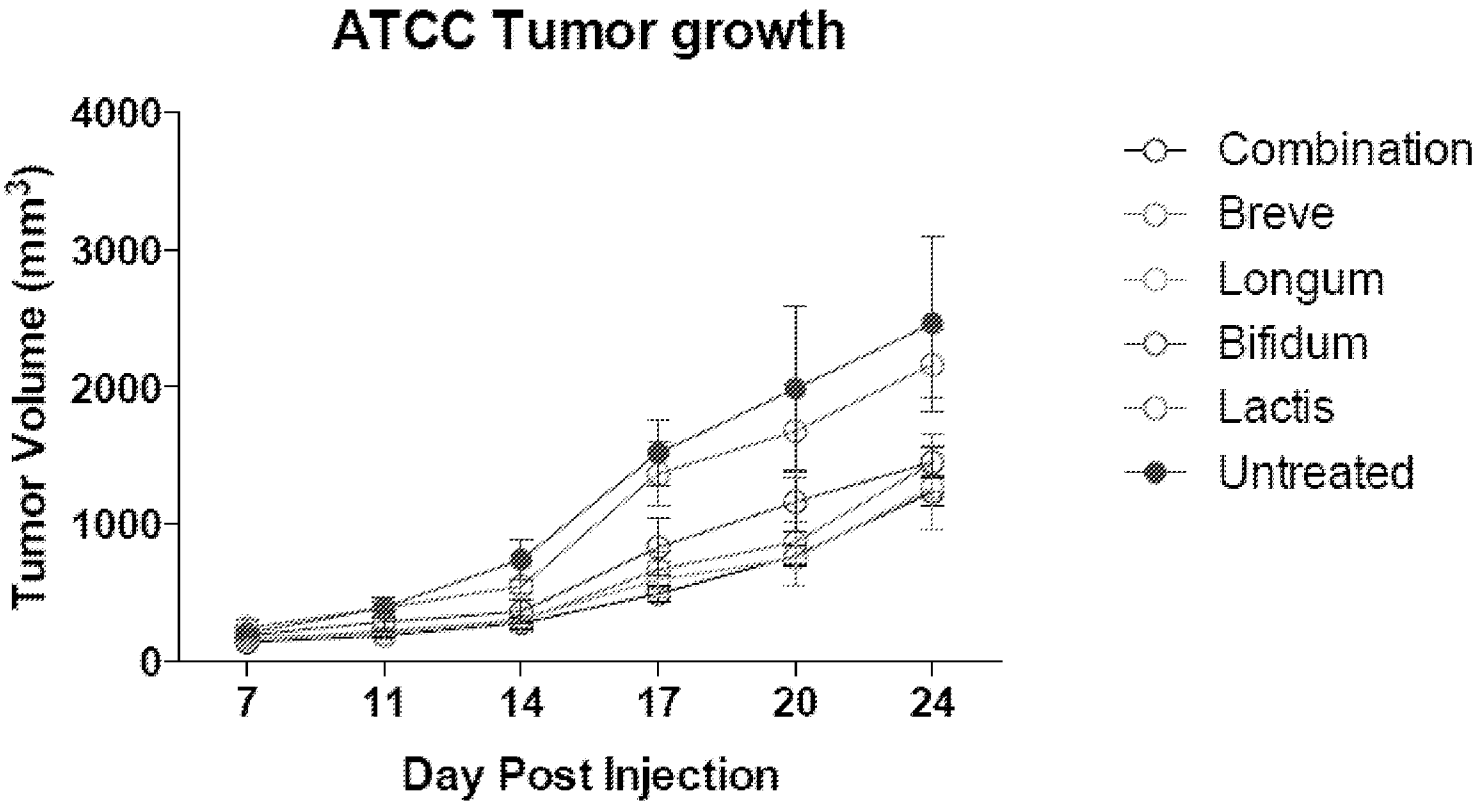


FIG. 15



PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 15/170,284

filed on 01-Jun-2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

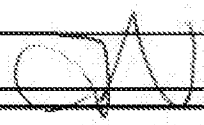
I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Thomas F. Gajewski Date (Optional): 6/21/16

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 15/170,284

filed on 01-Jun-2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

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LEGAL NAME OF INVENTOR

Inventor: Leticia Corrales Date (Optional): 6/21/2016

Signature: Leticia Corrales

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 15/170,284 filed on 01-Jun-2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Ayelet Sivan Date (Optional) :

Signature:

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

Electronic Patent Application Fee Transmittal

Application Number:	15170284
Filing Date:	01-Jun-2016
Title of Invention:	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA
First Named Inventor/Applicant Name:	Thomas F. Gajewski
Filer:	David William Staple
Attorney Docket Number:	UCHI-34458/US-3/ORD

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Late Filing Fee for Oath or Declaration	2051	1	70	70
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**70**

Electronic Acknowledgement Receipt

EFS ID:	26664446
Application Number:	15170284
International Application Number:	
Confirmation Number:	8885
Title of Invention:	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA
First Named Inventor/Applicant Name:	Thomas F. Gajewski
Customer Number:	72960
Filer:	David William Staple/Stephanie Filandrinos
Filer Authorized By:	David William Staple
Attorney Docket Number:	UCHI-34458/US-3/ORD
Receipt Date:	17-AUG-2016
Filing Date:	01-JUN-2016
Time Stamp:	15:27:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$70
RAM confirmation Number	1904
Deposit Account	504302
Authorized User	Staple, David

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	34458US3ORD_RCAP_8-17-16.pdf	81555 27ce1a5ac5559b2337c4bc076654416f959c1186	no	3
Warnings:					
Information:					
2	Drawings-other than black and white line drawings	34458-ORD-REPLACEMENT-FIGURES.pdf	4027999 d113813a903b8baf2d7ac5fd9df4d1f07c8d6576	no	46
Warnings:					
Information:					
3	Oath or Declaration filed	34458US3ORD_Exec_Decls_ALL.pdf	1110714 57c20acc4f921e1f2abbbe36fb44bb15307d6448	no	4
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30931 996865a98ed241fbe8830c0468bd7b43d624c4e4	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	5251199		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/170,284	06/01/2016	1653	1130	UCHI-34458/US-3/ORD	30	2

CONFIRMATION NO. 8885

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

FILING RECEIPT

OC000000083720389

Date Mailed: 06/17/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Thomas F. Gajewski, Chicago, IL;
Ayelet Sivan, Chicago, IL;
Leticia Corrales, Chicago, IL;

Applicant(s)

The University of Chicago, Chicago, IL;

Power of Attorney: The patent practitioners associated with Customer Number 72960

Domestic Priority data as claimed by applicant

This appln claims benefit of 62/169,112 06/01/2015
and claims benefit of 62/248,741 10/30/2015

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 06/15/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/170,284**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA

Preliminary Class

424

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/170,284

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	30 minus 20 =	* 10	x 40 =	400	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*	x 210 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	1130		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/170,284	06/01/2016	Thomas F. Gajewski	UCHI-34458/US-3/ORD

CONFIRMATION NO. 8885

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

FORMALITIES LETTER

OC000000083720370

Date Mailed: 06/17/2016

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - Numbers, letters, and reference characters on the drawings must measure at least 0.32 cm (1/8 inch) in height. See Figure(s) 1D, 2C, 3F, 7B, 8B, 9C, 12B, 13A, 14A.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. Small entity discount is in effect. If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **70** surcharge.
- $(**0**) previous unapplied payment amount.
- $ **70** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Thomas F. Gajewski
  Ayelet Sivan
  Leticia Corrales

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/bcao/

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
15/170,284	01-Jun-2016

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 72960

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

THE UNIVERSITY OF CHICAGO

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	
Name	ALAN THOMAS		
Title	ASSO. VP AND DIRECTOR, UCHICAGO TECH		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	26072198
Application Number:	15170284
International Application Number:	
Confirmation Number:	8885
Title of Invention:	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA
First Named Inventor/Applicant Name:	Thomas F. Gajewski
Customer Number:	72960
Filer:	David William Staple/Lisa M. Day
Filer Authorized By:	David William Staple
Attorney Docket Number:	UCHI-34458/US-3/ORD
Receipt Date:	15-JUN-2016
Filing Date:	01-JUN-2016
Time Stamp:	14:03:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	34458US3ORD_UCHI_EXE_POA.pdf	87265 fb70a30270370389d8532afd11d0bfe0571f5811	no	1

Warnings:	
Information:	

Total Files Size (in bytes):	87265

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 15170284 Document Date: 06/01/2016

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

At the time of document entry (noted above):

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- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Gajewski	Nonprovisional Application Number (if known):	
Title of Invention:	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

I. ☑ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. ☐ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /David W. Staple/	Date 2016-06-01
Name (Print/Typed) David W. Staple	Practitioner Registration Number 65903

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

☑ *Total of 1 forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Thomas	F.	Gajewski	

Residence Information (Select One) ● US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Chicago	IL	US

Mailing Address of Inventor:

Address 1	5801 S. Ellis Avenue
Address 2	
City	Chicago
State/Province	IL
Postal Code	60637
Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Ayelet		Sivan	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Chicago	IL	US

Mailing Address of Inventor:

Address 1	5801 S. Ellis Avenue
Address 2	
City	Chicago
State/Province	IL
Postal Code	60637
Country	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Leticia		Corrales	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

City	Chicago	State/Province	IL	Country of Residence	US

Mailing Address of Inventor:

Address 1	5801 S. Ellis Avenue		
Address 2			
City	Chicago	**State/Province**	IL
Postal Code	60637	**Country**	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	72960		
Email Address	docketing@casimirjones.com	Add Email	Remove Email

Application Information:

Title of the Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA		
Attorney Docket Number	UCHI-34458/US-3/ORD	**Small Entity Status Claimed**	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	38	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

PTO/AIA/14 (11-15)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	72960		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Claims benefit of provisional	62169112	2015-06-01
Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Claims benefit of provisional	62248741	2015-10-30

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 — Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	The University of Chicago		
Mailing Address Information For Applicant:			
Address 1	5801 S. Ellis Avenue		
Address 2			
City	Chicago	**State/Province**	IL
Country	US	Postal Code	60637
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

PTO/AIA/14 (11-15)
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Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/David W. Staple/			Date (YYYY-MM-DD)	2016-06-01
First Name	David W.	Last Name	Staple	Registration Number	65903

Additional Signature may be generated within this form by selecting the Add button. Add

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	UCHI-34458/US-3/ORD
	Application Number	
Title of Invention	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA	

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The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the priority benefit of U.S. Provisional Patent Application 62/169,112, filed June 1, 2015, and U.S. Provisional Patent Application 62/248,741, filed October 30, 2015, each of which is incorporated by reference in its entirety.

FIELD

Provided herein are methods of treatment and/or prevention of cancer by manipulation of commensal microflora. In particular, the amount, identity, presence, and/or ratio of microflora (e.g., gut microflora) in a subject is manipulated to facilitate one or more co-treatments.

BACKGROUND

Harnessing the host immune system constitutes a promising approach for the treatment of cancer because of its potential to specifically target tumor cells while limiting harm to normal tissue, with durability of benefit associated with immunologic memory. Enthusiasm has been fueled by recent clinical success, particularly with antibodies that block immune inhibitory pathways, specifically CTLA-4 and the PD-1/PD-L1 axis (Hodi et al. The New England journal of medicine 363, 711-723 (2010).; Hamid et al. The New England journal of medicine 369, 134-144 (2013).; herein incorporated by reference in their entireties). Early data have indicated that clinical responses to these immunotherapies are more frequent in patients who show evidence of an endogenous T cell response ongoing in the tumor microenvironment at baseline (Tumeh et al. Nature 515, 568-571 (2014).; Spranger et al. Science translational medicine 5, 200ra116 (2013).; Ji et al. Cancer immunology, immunotherapy : CII 61, 1019-1031 (2012).; Gajewski et al. Cancer journal 16, 399-403 (2010).; herein incorporated by reference in their entireties). Despite the functional and clinical importance of this T cell-inflamed tumor microenvironment, the mechanisms that govern the presence or absence of this phenotype have not been well understood. Theoretical sources of inter-patient heterogeneity include germline genetic differences at the level of the host, variability in patterns of somatic alterations in tumor cells, and environmental differences with the potential to impact on systemic immunity.

SUMMARY

Provided herein are methods of treatment and/or prevention of cancer by manipulation of commensal microflora. In particular, the amount, identity, presence, and/or ratio of microflora (e.g., gut microflora) in a subject is manipulated to facilitate one or more co-treatments.

In some embodiments, provided herein are methods of treating or preventing cancer in a subject, comprising modulating levels of one or more commensal microbes within the subject to: (A) enhance an immune response by the subject, (B) inhibit the growth or spread of the cancer, (C) inhibit immune evasion by the cancer, and/or (D) enhance the efficacy of a therapeutic. In some embodiments, the levels of one or more commensal microbes are modulated within the gut of the subject. In some embodiments, modulating the levels of one or more commensal microbes comprises increasing and/or decreasing levels of one or more bacterial selected from the genera *Adlercreutzia, Oscillopira, Mollicutes, Butyrivibrio, Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidobacterium, Rikenella, Alistipes, Marinilabilia, Anaerostipes, Escherichia*, and/or *Lactobacillus.*

In some embodiments, modulating the levels of one or more commensal microbes comprises administering a beneficial microbes to the subject. In some embodiments, the beneficial microbes are bacteria. In some embodiments, the bacteria are selected from the genera *Adlercreutzia, Oscillopira, Mollicutes, Butyrivibrio, Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidobacterium, Rikenella, Alistipes, Marinilabilia, Anaerostipes, Escherichia*, and/or *Lactobacillus.* In some embodiments, the bacteria are *Bifidobacterium*. In some embodiments, the *Bifidobacterium* include bacteria selected from the group consisting of *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis,* and *Bifidobacterium angulatum.* In some embodiments, the beneficial microbes are administered as a probiotic composition or via microflora transplant from a donor.

In some embodiments, modulating the levels of one or more commensal microbes comprises administering one or more antimicrobials. In some embodiments, the antimicrobial kills detrimental microbes. In some embodiments, the antimicrobial is an antibiotic. In some embodiments, methods further comprise administration of beneficial microbes to the subject.

In some embodiments, methods further comprise administering to the subject a cancer therapy. In some embodiments, wherein the modulating levels of one or more commensal microbes within the subject enhances an immune response by the subject and/or inhibits immune evasion by the cancer, and the cancer therapy is an immunotherapy. In some embodiments, the immunotherapy comprises administration of anti-CTLA-4 antibodies and/or anti-PD-L1 or anti-PD-1 antibodies. In some embodiments, wherein the modulating levels of one or more commensal microbes within the subject enhance the efficacy of a therapeutic, and the cancer therapy is said therapeutic. In some embodiments, the therapeutic comprises a chemotherapeutic. In some embodiments, methods further comprise testing the subject for immune evasion by the cancer. In some embodiments, methods further comprise surgical, radiation, and/or chemotherapeutic cancer intervention.

In some embodiments, provided herein are kits or compositions comprising a beneficial commensal microbe and a cancer therapeutic, said compositions or components of said kits formulated for therapeutic delivery to a subject.

In some embodiments, provided herein are beneficial commensal microbes for use as a medicament in the treatment of cancer and/or inhibition of immune evasion.

In some embodiments, provided herein are methods of treating or preventing cancer in a subject comprising administering to the subject bacterial formulation comprising bacteria of the genera *Bifidobacterium*, *Rikenella, Alistipes, Marinilabilia*, or *Anaerostipes*. In some embodiments, at least 50% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, *Rikenella, Alistipes, Marinilabilia*, or *Anaerostipes*. In some embodiments, at least 90% of the bacteria in the bacterial formulation are of the genera Bifidobacterium, *Rikenella, Alistipes, Marinilabilia*, or *Anaerostipes*. In some embodiments, the bacterial formulation comprise bacteria of the genus Bifidobacterium. In some embodiments, at least 50% of the bacteria in the bacterial formulation are of the genus Bifidobacterium. In some embodiments, at least 90% of the bacteria in the bacterial formulation are of the genus Bifidobacterium.

In some embodiments, the bacteria of genus Bifidobacterium are selected from the group consisting of *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis,*

Bifidobacterium angulatum, Bifidobacterium asteroides, Bifidobacterium boum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium cuniculi, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium indicum, Bifidobacterium inopinatum, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium pseudolongum, Bifidobacterium pullorum, Bifidobacterium psychraerophilum, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium scardovii, Bifidobacterium simiae, Bifidobacterium subtile, Bifidobacterium therammcidophilum, Bifidobacterium thermophilum, Bifidobacterium tsurumiense, Bifidobacterium urinalis, Bifidobacterium sp.

In some embodiments, the cancer is cancer is selected from the group consisting of acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemic leukemia, basophilic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, undifferentiated cell leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, promyelocytic leukemia, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiennoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform

carcinoma, gelatinous carcinoma, giant cell carcinoma, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, carcinoma villosum, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypernephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, naspharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, carcinoma scroti, chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, endometrial sarcoma, stromal sarcoma, Ewing' s sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms' tumor sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, telangiectaltic sarcoma, Hodgkin's Disease, Non-Hodgkin's Lymphoma, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insulanoma, malignant carcinoid, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant

hypercalcemia, cervical cancer, endometrial cancer, adrenal cortical cancer, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, nodular melanoma subungal melanoma, and superficial spreading melanoma.

In some embodiments, the subject is human. In some embodiments, the bacterial formulation is administered by oral administration, rectal administration, topical administration, inhalation or injection. In some embodiments, the bacterial formulation is a food product. In some embodiments, the bacterial formulation comprises at least about $5x10^6$ CFU of bacteria. In some embodiments, the bacterial formulation is administered to the subject in two or more doses. In some embodiments, the administration of at least two of the two or more doses are separated by at least 1 day. In some embodiments, the administration of at least two of the two or more doses are separated by at least 1 week.

In some embodiments, methods further comprise administering to the subject an antibiotic. In some embodiments, the antibiotic is administered to the subject before the bacterial formulation. In some embodiments, the antibiotic is administered to the subject at least 1 day before the bacterial formulation is administered to the subject.

In some embodiments, methods further comprise administering to the subject an immune checkpoint inhibitor. In some embodiments, the immune checkpoint inhibitor is a protein or polypeptide that specifically binds to an immune checkpoint protein. In some embodiments, the immune checkpoint protein is selected from the group consisting of CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA. In some embodiments, the polypeptide or protein is an antibody or antigen-binding fragment thereof. In some embodiments, the immune checkpoint inhibitor is an interfering nucleic acid molecule. In some embodiments, the interfering nucleic acid molecule is an siRNA molecule, an shRNA molecule or an antisense RNA molecule. In some embodiments, the immune checkpoint inhibitor is selected from the group consisting of nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT Ol l, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010. In some embodiments, the immune checkpoint inhibitor is administered before the bacterial formulation. In some embodiments, the immune checkpoint inhibitor is administered at least one day before

the bacterial formulation. In some embodiments, the immune checkpoint is administered at about the same time as the bacterial formulation. In some embodiments, the immune checkpoint inhibitor is administered on the same day as the bacterial formulation. In some embodiments, the immune checkpoint inhibitor is administered after the bacterial formulation. In some embodiments, the immune checkpoint inhibitor is administered at least one day after the bacterial formulation. In some embodiments, the immune checkpoint inhibitor is administered by injection. In some embodiments, the injection is an intravenous, intramuscular, intratumoral or subcutaneous injection.

In some embodiments, provided herein are methods of treating cancer in a human subject comprising administering to the subject an immune checkpoint inhibitor and a bacterial formulation comprising bacteria of the genera *Bifidobacterium*. In some embodiments, at least 50% (e.g., 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or more, or ranges therebetween) of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*. In some embodiments, at least 90% (e.g., 90%, 95%, 99%, 99.9%, 99.99%, or more or ranges therebetween) of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*. In some embodiments, the bacteria of the genus *Bifidobacterium* comprise bacteria of the species *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium asteroides, Bifidobacterium boum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium cuniculi, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium indicum, Bifidobacterium inopinatum, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium pseudolongum, Bifidobacterium pullorum, Bifidobacterium psychraerophilum, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium scardovii, Bifidobacterium simiae, Bifidobacterium subtile, Bifidobacterium therammcidophilum, Bifidobacterium thermophilum, Bifidobacterium tsurumiense, Bifidobacterium urinalis* or *Bifidobacterium sp.* In some embodiments, the bacterial formulation is administered by oral administration or rectal administration. In some embodiments, the bacterial formulation is administered by oral administration. In some embodiments, the bacterial formulation comprises at least $5x10^6$ CFU (e.g., $5x10^6$ CFU, $1x10^7$ CFU, $2x10^7$ CFU, $5x10^7$

CFU, $1x10^8$ CFU, $2x10^8$ CFU, $5x10^8$ CFU, $1x10^9$ CFU, $2x10^9$ CFU, $5x10^9$ CFU, $1x10^{10}$ CFU, $2x10^{10}$ CFU, $5x10^{10}$ CFU, $1x10^{11}$ CFU, $2x10^{11}$ CFU, $5x10^{11}$ CFU, $1x10^{12}$ CFU, $2x10^{12}$ CFU, $5x10^{12}$ CFU, or more or ranges therebetween) of bacteria of the genera *Bifidobacterium*. In some embodiments, the bacterial formulation is administered to the subject in two or more doses (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, or ranges therebetween). In some embodiments, the administration of doses are separated by at least 1 week. In some embodiments, methods further comprise administering to the subject an antibiotic prior to the administration of the bacterial formulation. In some embodiments, the antibiotic is administered to the subject at least 1 day before the bacterial formulation is administered to the subject. In some embodiments, the immune checkpoint inhibitor is a protein or polypeptide that binds to an immune checkpoint protein. In some embodiments, the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to an immune checkpoint protein. In some embodiments, the immune checkpoint protein is CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA. In some embodiments, the immune checkpoint protein is PD-1 or PD-L1. In some embodiments, the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT Ol l, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010. In some embodiments, the immune checkpoint inhibitor is administered by intravenous injection, intramuscular injection, intratumoral injection or subcutaneous injection.

In some embodiments, provided herein are methods of treating cancer in a human subject comprising administering to the subject a bacterial formulation comprising at least $5x10^6$ CFU (e.g., $5x10^6$ CFU, $1x10^7$ CFU, $2x10^7$ CFU, $5x10^7$ CFU, $1x10^8$ CFU, $2x10^8$ CFU, $5x10^8$ CFU, $1x10^9$ CFU, $2x10^9$ CFU, $5x10^9$ CFU, $1x10^{10}$ CFU, $2x10^{10}$ CFU, $5x10^{10}$ CFU, $1x10^{11}$ CFU, $2x10^{11}$ CFU, $5x10^{11}$ CFU, $1x10^{12}$ CFU, $2x10^{12}$ CFU, $5x10^{12}$ CFU, or more or ranges therebetween) of bacteria of the genera *Bifidobacterium,* wherein at least 50% (e.g., 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or more, or ranges therebetween) of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*. In some embodiments, at least 90% (e.g., 90%, 95%, 99%, 99.9%, 99.99%, or more or ranges therebetween) of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*. In some embodiments, the bacteria of the genus *Bifidobacterium* comprise bacteria of the species *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium*

breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium asteroides, Bifidobacterium boum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium cuniculi, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium indicum, Bifidobacterium inopinatum, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium pseudolongum, Bifidobacterium pullorum, Bifidobacterium psychraerophilum, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium scardovii, Bifidobacterium simiae, Bifidobacterium subtile, Bifidobacterium therammcidophilum, Bifidobacterium thermophilum, Bifidobacterium tsurumiense, Bifidobacterium urinalis or *Bifidobacterium sp.* In some embodiments, the bacterial formulation is administered by oral administration or rectal administration. In some embodiments, the bacterial formulation is administered by oral administration. In some embodiments, the bacterial formulation is administered to the subject in two or more doses (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, or ranges therebetween). In some embodiments, methods further comprise administering to the subject an antibiotic before the bacterial formulation is administered to the subject. In some embodiments, methods further comprise administering to the subject an immune checkpoint inhibitor. In some embodiments, the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA. In some embodiments, the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to PD-1 or PD-L1. In some embodiments, the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT Ol l, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A-H. Differences in melanoma outgrowth and tumor-specific immune responses between C57BL/6 JAX and TAC mice are eliminated upon cohousing. (A) B16.SIY tumor growth kinetics in newly arrived JAX and TAC mice. (B) IFN-γ ELISPOT in tumor-bearing JAX and TAC mice 7 days following tumor inoculation. (C) Mean size of IFN-γ spots (10^{-3}

mm^2). (D) Percentage of SIY^+ T cells of total $CD8^+$ T cells within the tumor of JAX and TAC mice as determined by flow cytometry 21 days post-tumor inoculation. Representative plots (left), quantification (right). (E) B16.SIY tumor growth kinetics in JAX and TAC mice cohoused for 3 weeks prior to tumor inoculation. (F) Number of IFN-γ spots/10^6 splenocytes in tumor-bearing JAX and TAC mice cohoused for 3 weeks prior to tumor inoculation. (G) Mean size of IFN-γ spots (10^{-3} mm^2). (H) Percentage of SIY^+ T cells of total $CD8^+$ T cells within the tumor of JAX and TAC mice cohoused for 3 weeks prior to tumor inoculation.

Fig. 2A-G. Oral administration of JAX fecal material to TAC mice enhances spontaneous anti-tumor immunity and response to αPD-L1 mAb therapy. (A) B16.SIY tumor growth in newly arrived TAC mice, TAC and JAX mice orally gavaged with PBS, TAC or JAX fecal material prior to tumor implantation. (B) Number of IFN-γ spots x mean spot size (10^{-3} mm^2), determined by ELISPOT 7 days following tumor inoculation. (C) Percentage of SIY^+ $CD8^+$ T cells within the tumor of TAC and JAX mice treated as in (A), 21 days post-tumor inoculation. Representative plots (left), quantification (right). (D) B16.SIY tumor growth in TAC mice, untreated or treated with JAX fecal material 7 and 14 days post tumor implantation, αPD-L1 mAb 7, 10, 13 and 16 days post tumor implantation, or both regimens. (E) IFN-γ ELISPOT assessed 5 days after start of treatment. (F) Percentage of tumor-infiltrating SIY^+ $CD8^+$ T cells, determined by flow cytometry 14 days after start of treatment. (G) B16.SIY tumor growth kinetics in TAC and JAX mice, untreated or treated with αPD-L1 mAb 7, 10, 13 and 16 days post tumor implantation.

Fig. 3A-G. Direct administration of *Bifidobacterium* to TAC recipients with established tumors improves tumor-specific immunity and response to αPD-L1 mAb therapy. (A-C) Bacterial species diversity (A), principal coordinate analysis plot of bacterial β-diversity (B) and operational taxonomic unit (OTU) levels of top *Bifidobacterium* taxon (C) in fecal material obtained from JAX, TAC, TAC-fed TAC and JAX-fed TAC mice. Comparisons in A-C were performed using 9-10 replicates from each vendor and 4-5 replicates from each treatment. (D) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation (white arrows), αPD-L1 mAb 7, 10, 13 and 16 days post tumor implantation (black arrows) or both regimens. (E) IFN-γ ELISPOT assessed 5 days after start of treatment. (F) Percentage of tumor-infiltrating SIY^+ $CD8^+$ T cells, determined by flow

cytometry 14 days following start of treatment. Representative plots (left), quantification of data combined from 2 independent experiments (right). (G) B16.SIY tumor growth for isotype-treated (left) or CD8-depleted (right) groups as in D.

Fig. 4A-E. Dendritic cells isolated from JAX and *Bifidobacterium*-fed TAC mice show increased expression of genes associated with antitumor immunity and heightened capability for T cell activation (A) Quantification of IFN-γ MFI (mean fluorescence intensity) of 2C $CD8^+$ T cells in the tumor-draining lymph node (left) and spleen (right) of TAC, JAX, *Bifidobacterium*-fed TAC mice on day 7 post adoptive transfer. (B) Percentage of MHC Class IIhi DCs in tumors isolated from TAC, JAX, and *Bifidobacterium*-fed TAC mice 40 hours post tumor implantation as assessed by flow cytometry. (C) Enriched biological pathways and functions found within the subset of elevated genes in JAX and *Bifidobacterium*-treated TAC-derived DCs relative to untreated TAC DCs isolated from tumors 40hrs post tumor inoculation, as assessed by DAVID pathway analysis. Bars indicate the percent of genes in a pathway upregulated in DCs isolated from JAX and *Bifidobacterium*-fed TAC mice. Line indicates p-values calculated by Fisher's exact test. (D) Heat map of key antitumor immunity genes in DCs isolated from JAX, *Bifidobacterium*-treated TAC or untreated TAC mice. Mean fold-change for each gene transcript is shown on the right. (E) Quantification of IFN-γ^+ 2C TCR Tg $CD8^+$ T cells stimulated in vitro with DCs purified from peripheral lymphoid tissues of naïve TAC, JAX, and *Bifidobacterium*-treated TAC mice in the presence of different concentrations of SIY peptide.

Fig. 5A-D. (A) Schematic of prophylactic fecal transfer: fecal pellets collected from JAX and TAC mice upon arrival in our facility were resuspended in PBS, homogenized and the supernatant was introduced by oral gavage into either JAX or TAC recipients as shown, once a week for two weeks prior to B16.SIY tumor inoculation. (B) B16.SIY tumor growth in JAX mice orally gavaged with TAC or JAX fecal material once weekly for two weeks prior to tumor implantation. (C) Percentage of SIY^+ T cells of total $CD8^+$ T cells within the tumor of groups as in Figure 2A, determined by flow cytometry 7 days post-tumor inoculation. (D) Percentage of SIY^+ T cells of total $CD8^+$ T cells within the tumor of JAX and TAC mice, untreated or treated with αPD-L1 mAb, as determined by flow cytometry 21 days post-tumor inoculation.

Fig. 6A-H. (A) Relative abundance of all taxa combined belonging to the *Bifidobacterium* genus in fecal material obtained from TAC, JAX, TAC-fed TAC and JAX-fed TAC mice. Comparisons were performed using 9-10 replicates from each vendor and 4-5

replicates from each treatment. (B) Number of colony forming units (CFU) of live and heat inactivated bifidobacteria, plated in RCM agar following serial dilution in reduced PBS and incubated in an anaerobic chamber for 72 hours. (C) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with live *Bifidobacterium*, heat inactivated *Bifidobacterium* or JAX fecal material 7 and 14 days post tumor implantation. (D) Percentage of tumor-infiltrating SIY^+ T cells of total $CD8^+$ T cells for treatment groups as in C, determined by flow cytometry 14 days after start of treatment. C-D show data combined from 2-4 independent experiments, 5 mice per group. (E) B16.F10 tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation. (F) MB49 tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation. (G) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with *Lactobacillus murinus* or JAX fecal material 7 and 14 days post tumor implantation. (H) Percentage of tumor-infiltrating SIY^+ T cells of total $CD8^+$ T cells for treatment groups as in G, determined by flow cytometry 18 days after start of treatment.

Fig. 7A-B. (A) Schematic of *in vivo* 2C proliferation assays. CD8+ T cells were isolated from the spleen and lymph node of naïve 2C TCR Tg CD45.1+/.2+mice, labeled with CFSE and injected i.v. into CD45.2+ C57BL/6 mice derived from either TAC, JAX or *Bifidobacterium*-treated TAC mice. 24 hours later, mice were inoculated with 1x106 B16.SIY melanoma cells s.c. Spleen and tumor-draining lymph node were harvested and restimulated ex-vivo with SIY peptide. Intracellular IFN-γ production and CFSE dilution were assessed in gated CD45.1+/.2+ 2C T cells by flow cytometry; TDLN=tumordraining lymph node. (B) Representative CFSE dilution assessed in gated CD45.1+/.2+ 2C T cells by flow cytometry (left) and quantification (right).

Fig. 8A-G. Direct administration of *Bifidobacterium* to TAC recipients with established tumors improves tumor-specific immunity and response to αPD-L1 mAb therapy. (A) Principal coordinate analysis plot of bacterial β-diversity over time in groups treated as in Figure 2A. (B) Phylogenetic analysis of taxa that are of significantly different abundance in newly arrived JAX vs TAC mice FDR<0.05 (non-parametric *t* test); bars represent log-transformed fold changes, inner circle=log10(10); middle circle=log10(100); outer circle=log10(1000). (C) Heatmap demonstrating relative abundance over time of significantly altered genus-level taxa in JAX-fed TAC mice FDR<0.05 (non-parametric *t* test); columns depict individual mice; each timepoint

shows mice from two separate cages, 3-4 mice per cage. (D) Correlation plot of relative abundance of *Bifidobacterium* OTU_681370 in fecal material obtained from groups as in (A) 14 days post arrival and frequency of SIY^+ $CD8^+$ T cells in tumor; p=1.4x10-5, FDR=0.0002, R2=0.86 (univariate regression). (E) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation, αPD-L1 mAb 7, 10, 13 and 16 days post tumor implantation, or both regimens. (F) IFN-γ ELISPOT assessed 5 days after start of treatment. (G) Percentage of tumor-infiltrating SIY^+ $CD8^+$ T cells, determined by flow cytometry 14 days following start of treatment.

Fig. 9A-E. (A) Relative abundance of *Bifidobacterium* OTU_681370 in fecal material obtained from TAC mice 7 days following inoculation with commercial *Bifidobacterium* species. (B) *Bifidobacterium* levels in fecal material obtained from groups as shown, assessed by qPCR using genus-specific primers. (C) Representative plots showing percentage of SIY^+ T cells of total $CD8^+$ T cells within the tumor of untreated and *Bifidobacterium*-treated TAC mice, as assessed by flow cytometry 14 days following start of treatment. (D) *Bifidobacterium* levels in TAC mice 3 weeks post *Bifidobacterium* administration, assessed by qPCR. (E) B16.SIY tumor growth in TAC mice, untreated or inoculated with *Bifidobacterium* 6 weeks prior to tumor implantation.

Fig. 10A-D. (A) B16.SIY tumor growth for isotype-treated (left) or CD8-depleted (right) groups as in Figure 3E. (B) Number of colony forming units (CFU) of live and heat inactivated bifidobacteria, plated in RCM agar following serial dilution in reduced PBS and incubated in an anaerobic chamber for 72 hours. Bars represent 2 replicate plates of each dilution. (C) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with live *Bifidobacterium*, heat inactivated *Bifidobacterium* or JAX fecal material 7 and 14 days post tumor implantation. (D) Percentage of tumor-infiltrating SIY^+ T cells of total $CD8^+$ T cells for treatment groups as in (C), determined by flow cytometry 14 days after start of treatment.

Fig. 11A-E. (A) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with ATCCderived *B. breve* or *B. longum.* (B) B16.F10 tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation. (C) MB49 tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation. (D) B16.SIY tumor growth kinetics in TAC mice, untreated or treated with *Lactobacillus murinus* or JAX fecal material 7 and 14 days post tumor implantation. (E)

Percentage of tumor-infiltrating SIY^+ T cells of total $CD8^+$ T cells for treatment groups as in (D), determined by flow cytometry 18 days after start of treatment.

Fig. 12A-C. (A) Heatmap demonstrating relative abundance of significantly altered genus-level taxa in *Bifidobacterium*-fed TAC mice FDR<0.05 (non-parametric *t*-test); columns depict individual mice; *n = 4-8* mice per group. (B) Frequency of CD4+ FOXP3+ T cells in tumors isolated from JAX and TAC mice 21 days post tumor inoculation, assessed by flow cytometry; representative plot (top), quantification (bottom). (C) Evaluation of translocation of *Bifidobacterium* into mesenteric lymph nodes (mLN), spleen and tumor of TAC, JAX and *Bifidobacterium*-inoculated mice, assessed by qPCR.

Fig. 13A-C. (A) Representative plots depicting the strategy for isolation of DCs from tumors in JAX, TAC and *Bifidobacterium*-treated TAC mice: live CD45+CD3-CD19-MHCIIhiCD11c+ dendritic cells were sorted as shown. (B) All enriched biological pathways and functions found within the subset of elevated genes (fold change ≥ 1.5) in JAX and *Bifidobacterium*-treated TAC-derived DCs relative to untreated TAC DCs isolated from tumors 40hrs post inoculation, as assessed by DAVID pathway analysis. (C) qPCR validation of genes identified by microarray gene expression profiling as in (B).

Fig. 14A-B. (A) Representative flow plots of CFSE dilution and IFN-γ production in 2C CD8+ T cells stimulated in vitro with DCs purified from naive TAC, JAX and *Bifidobacterium*-treated TAC mice in the presence of different concentrations of SIY peptide as shown. (B) Percentage of 2C CD8+ T cells with undiluted CFSE, stimulated in vitro with DCs purified from naive TAC, JAX and *Bifidobacterium*-treated TAC mice in the presence of different concentrations of SIY peptide as shown.

Fig. 15. B16.SIY tumor growth in TAC mice, untreated or treated individually with ATCC 15700 *B. breve,* ATCC BAA-999 *B. longum,* ATCC 27536 *B. Lactis* or ATCC 15696 *B. Bifidum,* or treated with all four strains combined.

DEFINITIONS

As used herein, the term "microbe" refers to cellular microorganisms including bacteria, fungi, and archaea, and encompasses both individual organisms and populations comprising any number of the organisms.

As used herein, the term "microflora" refers to an assemblage of microorganisms localized to a distinct environment. Microflora may include, for example, populations of various bacteria, fungi, and/or archaea that inhabit a particular environment. For example, "gut microflora," "vaginal microbiota," and "oral microflora" are an assemblage of one or more species of microorganisms that are localized to, or found in, the gut, vagina, or mouth, respectively. "Normal microflora" refers to a population of microorganisms that localize in a particular environment in a normal, non-pathological state (e.g., a sample of gut microflora from a subject without cancer). "Pathologic microflora" refers to a population of various microorganisms that localize in a particular environment in pathological state and differs from normal microflora in terms of identify, absolute amount, or relative amount of the various microbes.

As used herein, the term "commensal microbe" refers to a microorganism that is non-pathogenic to a host and is part of the normal microflora of the host.

As used herein, the term "co-administration" refers to the administration of at least two agents (e.g., commensal microflora and a cancer therapy) or therapies to a subject. In some embodiments, the co-administration of two or more agents/therapies is concurrent. In other embodiments, the co-administration of two or more agents/therapies is sequential (e.g., a first agent/therapy is administered prior to a second agent/therapy).

As used herein, the term "beneficial microbe" refers to a microbe (e.g., bacterium) strain or species that inhibits the growth of cancer/tumor cells and/or facilitates treatment of cancer/tumor cells (e.g., inhibits immune evasion). Beneficial microbes may function by, for example, creating an anti-cancer/anti-tumor environment, microenvironment and/or metabolome, and/or by creating an environment, microenvironment and/or metabolome that inhibits immune evasion or other mechanisms by which cancer cells resist therapy.

As used herein, the term "detrimental microbe" refers to a microbe (e.g., bacterium) strain or species that facilitates the growth of cancer/tumor cells and/or prevents or reduces the effectiveness of treatment of cancer/tumor cells (e.g., inhibits immune evasion). Detrimental microbes may function by, for example, creating an environment, microenvironment and/or metabolome that facilitates immune evasion or other mechanisms by which cancer cells resist therapy and/or enhance cancer/tumor growth.

As used herein, the term "pharmaceutical agent" refers to a compound, macromolecule, or other chemical/non-biological entity that is administered to a subject to elicit a desired biological response. A pharmaceutical agent may be a "drug" or another entity which is biologically active in a human being or other mammal, locally and/or systemically. Examples of drugs are disclosed in the Merck Index and the Physicians Desk Reference, the entire disclosures of which are incorporated by reference herein for all purposes.

As used herein, the terms "microbial agent," "commensal microbial agent," and "probiotic" refer to compositions comprising a microbe or population of multiple different microbes for administration to a subject.

As used herein, the term "antimicrobial agent" is used to describe a therapeutic compound or bioactive agent which treats a microbial infection, for example, an infection caused by a bacteria, virus, protozoa or fungus. The antimicrobial agent may be an antibiotic, an antifungal agent, an antiviral or an antiprotozoal or antiparasitic agent (which may also be used to treat multicellular parasites).

As used herein, the terms "antibiotic" and "antibacterial agent" refer to a chemical agent which is active against bacteria. In common usage, an antibiotic is a substance or compound (also called chemotherapeutic agent) that kills or inhibits the growth of bacteria. Anti-bacterial antibiotics can be categorized based on their target specificity: "narrow-spectrum" antibiotics target particular types of bacteria, such as Gram-negative or Gram-positive bacteria, while broad-spectrum antibiotics affect a wide range of bacteria. Antibiotics which target the bacterial cell wall (e.g., penicillins, cephalosporins, cephems), or cell membrane (e.g., polymixins), or interfere with essential bacterial enzymes (e.g., quinolones, sulfonamides) usually are bactericidal in nature. Those which target protein synthesis such as the aminoglycosides, macrolides and tetracyclines are usually bacteriostatic. Three newer classes of antibiotics include: cyclic lipopeptides (e.g., daptomycin), glycylcyclines (e.g., tigecycline), and oxazolidinones (e.g., linezolid). Tigecycline is a broad-spectrum antibiotic, while the two others are useful for Gram-positive infections.

As used herein, the term "antiviral agent" refers to a chemical agent which is used to treat a viral infection. Antiviral drugs are a class of medication used specifically for treating viral infections, specific antivirals are useful for treating infection by specific viruses. Antivirals typically only inhibit virus development.

As used herein, the term "antifungal agent" refers to a therapeutic compound or bioactive agent which may be used to treat a fungal infection in a patient. An antifungal drug is a medication used to treat fungal infections such as athlete's foot, ringworm, candidiasis (thrush), serious systemic infections such as cryptococcal meningitis, and related fungal infections. Antifungal agents include, for example, polyene antifungals, imidazole, triazole and thiazole antifungals, allylamines, echinocandins, griseofulvin, flycystosine, undecylenic acid, among others.

As used herein, the term "antiparasitic agent" refers to a therapeutic compound or bioactive agent that is used to treat parasitic diseases including nematodes, cestodes, trematodes, infectious protozoa, and amoebas. Exemplary antiparasitic agents include: antinematodes (e.g., mebendazole, pyrantel pamoate, thiabendazole, diethycarbazine), anticestodes (e.g., niclosamide, praziquantel), antitrematodes (e.g., praziquantel), antiamoebics (e.g., rifampin and amphotericin B), antiprotozoals (e.g., melarsoprol, eflornithine, metronidazole and tinidazole), among others.

As used herein, the term "pharmaceutical formulation" refers to at least one pharmaceutical agent and/or microbial agent in combination with one or more additional components that assist in rendering the agent(s) suitable for achieving the desired effect upon administration to a subject. The pharmaceutical formulation may include one or more additives, for example pharmaceutically acceptable excipients, carriers, penetration enhancers, coatings, stabilizers, buffers or other materials physically associated with the pharmaceutical/microbial agent to enhance the administration, release (e.g., timing of release), deliverability, bioavailability, effectiveness, etc. of the dosage form. The formulation may be, for example, a liquid, a suspension, a solid, a nanoparticle, emulsion, micelle, ointment, gel, emulsion, coating, etc. A pharmaceutical formulation may contain a single agent or multiple agents (e.g., microbial agent and pharmaceutical agent).

As used herein, the term "subject" broadly refers to any animal, including but not limited to, human and non-human animals (e.g., dogs, cats, cows, horses, sheep, poultry, fish, crustaceans, etc.). As used herein, the term "patient" typically refers to a subject that is being treated for a disease or condition (e.g., cancer, solid tumor cancer, non-T cell-infiltrated tumor cancer, etc.).

As used herein, an "immune response" refers to the action of a cell of the immune system (e.g., T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, eosinophils, mast

cells, dendritic cells, neutrophils, etc.) and soluble macromolecules produced by any of these cells or the liver (including Abs, cytokines, and complement) that results in selective targeting, binding to, damage to, destruction of, and/or elimination from a subject of invading pathogens, cells or tissues infected with pathogens, or cancerous or other abnormal cells.

As used herein, the term "immunoregulator" refers to an agent or a signaling pathway (or a component thereof) that regulates an immune response. "Regulating," "modifying" or "modulating" an immune response refers to any alteration of the immune system or in the activity of such cell. Such regulation includes stimulation or suppression of the immune system which may be manifested by an increase or decrease in the number of various cell types, an increase or decrease in the activity of these cells, or any other changes which can occur within the immune system. Both inhibitory and stimulatory immunoregulators have been identified, some of which may have enhanced function in a cancer microenvironment.

As used herein, the term "immune evasion" refers to inhibition of a subject's immune system or a component thereof (e.g., endogenous T cell response) by a cancer or tumor cell in order to maximize or allow continued growth or spread of the cancer/tumor.

As used herein, the term "immunotherapy" refers to the treatment or prevention of a disease or condition (e.g., cancer) by a method comprising inducing, enhancing, suppressing or otherwise modifying an immune response.

As used herein, "potentiating an endogenous immune response" means increasing the effectiveness or potency of an existing immune response in a subject. This increase in effectiveness and potency may be achieved, for example, by overcoming mechanisms that suppress the endogenous host immune response or by stimulating mechanisms that enhance the endogenous host immune response.

As used herein, the term "antibody" refers to a whole antibody molecule or a fragment thereof (e.g., fragments such as Fab, Fab', and F(ab')2), it may be a polyclonal or monoclonal antibody, a chimeric antibody, a humanized antibody, a human antibody, etc.

A native antibody typically has a tetrameric structure. A tetramer typically comprises two identical pairs of polypeptide chains, each pair having one light chain (in certain embodiments, about 25 kDa) and one heavy chain (in certain embodiments, about 50-70 kDa). In a native antibody, a heavy chain comprises a variable region, VH, and three constant regions, CH1, CH2, and CH3. The VH domain is at the amino-terminus of the heavy chain, and the CH3 domain is at

the carboxy-terminus. In a native antibody, a light chain comprises a variable region, VL, and a constant region, CL. The variable region of the light chain is at the amino-terminus of the light chain. In a native antibody, the variable regions of each light/heavy chain pair typically form the antigen binding site. The constant regions are typically responsible for effector function.

In a native antibody, the variable regions typically exhibit the same general structure in which relatively conserved framework regions (FRs) are joined by three hypervariable regions, also called complementarity determining regions (CDRs). The CDRs from the two chains of each pair typically are aligned by the framework regions, which may enable binding to a specific epitope. From N-terminus to C-terminus, both light and heavy chain variable regions typically comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The CDRs on the heavy chain are referred to as H1, H2, and H3, while the CDRs on the light chain are referred to as L1, L2, and L3. Typically, CDR3 is the greatest source of molecular diversity within the antigen-binding site. H3, for example, in certain instances, can be as short as two amino acid residues or greater than 26. The assignment of amino acids to each domain is typically in accordance with the definitions of Kabat et al. (1991) Sequences of Proteins of Immunological Interest (National Institutes of Health, Publication No. 91-3242, vols. 1-3, Bethesda, Md.); Chothia, C., and Lesk, A. M. (1987) J. Mol. Biol. 196:901-917; or Chothia, C. et al. Nature 342:878-883 (1989). In the present application, the term "CDR" refers to a CDR from either the light or heavy chain, unless otherwise specified.

As used herein, the term "heavy chain" refers to a polypeptide comprising sufficient heavy chain variable region sequence to confer antigen specificity either alone or in combination with a light chain.

As used herein, the term "light chain" refers to a polypeptide comprising sufficient light chain variable region sequence to confer antigen specificity either alone or in combination with a heavy chain.

As used herein, when an antibody or other entity "specifically recognizes" or "specifically binds" an antigen or epitope, it preferentially recognizes the antigen in a complex mixture of proteins and/or macromolecules, and binds the antigen or epitope with affinity which is substantially higher than to other entities not displaying the antigen or epitope. In this regard, "affinity which is substantially higher" means affinity that is high enough to enable detection of an antigen or epitope which is distinguished from entities using a desired assay or measurement

apparatus. Typically, it means binding affinity having a binding constant (Ka) of at least 10^7 M^{-1} (e.g., $>10^7$ M^{-1}, $>10^8$ M^{-1}, $>10^9$ M^{-1}, $>10^{10}$ M^{-1}, $>10^{11}$ M^{-1}, $>10^{12}$ M^{-1}, $>10^{13}$ M^{-1}, etc.). In certain such embodiments, an antibody is capable of binding different antigens so long as the different antigens comprise that particular epitope. In certain instances, for example, homologous proteins from different species may comprise the same epitope.

As used herein, the term "monoclonal antibody" refers to an antibody which is a member of a substantially homogeneous population of antibodies that specifically bind to the same epitope. In certain embodiments, a monoclonal antibody is secreted by a hybridoma. In certain such embodiments, a hybridoma is produced according to certain methods known to those skilled in the art. See, e.g., Kohler and Milstein (1975) Nature 256: 495-499; herein incorporated by reference in its entirety. In certain embodiments, a monoclonal antibody is produced using recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In certain embodiments, a monoclonal antibody refers to an antibody fragment isolated from a phage display library. See, e.g., Clackson et al. (1991) Nature 352: 624-628; and Marks et al. (1991) J. Mol. Biol. 222: 581-597; herein incorporated by reference in their entireties. The modifying word "monoclonal" indicates properties of antibodies obtained from a substantially-homogeneous population of antibodies, and does not limit a method of producing antibodies to a specific method. For various other monoclonal antibody production techniques, see, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); herein incorporated by reference in its entirety.

As used herein, the term "antibody fragment" refers to a portion of a full-length antibody, including at least a portion antigen binding region or a variable region. Antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, Fv, scFv, Fd, diabodies, and other antibody fragments that retain at least a portion of the variable region of an intact antibody. See, e.g., Hudson et al. (2003) Nat. Med. 9:129-134; herein incorporated by reference in its entirety. In certain embodiments, antibody fragments are produced by enzymatic or chemical cleavage of intact antibodies (e.g., papain digestion and pepsin digestion of antibody) produced by recombinant DNA techniques, or chemical polypeptide synthesis.

For example, a "Fab" fragment comprises one light chain and the CH1 and variable region of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. A "Fab'" fragment comprises one light chain and one heavy chain

that comprises additional constant region, extending between the CH1 and CH2 domains. An interchain disulfide bond can be formed between two heavy chains of a Fab' fragment to form a "F(ab')2" molecule.

An "Fv" fragment comprises the variable regions from both the heavy and light chains, but lacks the constant regions. A single-chain Fv (scFv) fragment comprises heavy and light chain variable regions connected by a flexible linker to form a single polypeptide chain with an antigen-binding region. Exemplary single chain antibodies are discussed in detail in WO 88/01649 and U.S. Pat. Nos. 4,946,778 and 5,260,203; herein incorporated by reference in their entireties. In certain instances, a single variable region (e.g., a heavy chain variable region or a light chain variable region) may have the ability to recognize and bind antigen.

Other antibody fragments will be understood by skilled artisans.

As used herein, the term "chimeric antibody" refers to an antibody made up of components from at least two different sources. In certain embodiments, a chimeric antibody comprises a portion of an antibody derived from a first species fused to another molecule, e.g., a portion of an antibody derived from a second species. In certain such embodiments, a chimeric antibody comprises a portion of an antibody derived from a non-human animal fused to a portion of an antibody derived from a human. In certain such embodiments, a chimeric antibody comprises all or a portion of a variable region of an antibody derived from a non-human animal fused to a constant region of an antibody derived from a human.

A "humanized" antibody refers to a non-human antibody that has been modified so that it more closely matches (in amino acid sequence) a human antibody. A humanized antibody is thus a type of chimeric antibody. In certain embodiments, amino acid residues outside of the antigen binding residues of the variable region of the non-human antibody are modified. In certain embodiments, a humanized antibody is constructed by replacing all or a portion of a complementarity determining region (CDR) of a human antibody with all or a portion of a CDR from another antibody, such as a non-human antibody, having the desired antigen binding specificity. In certain embodiments, a humanized antibody comprises variable regions in which all or substantially all of the CDRs correspond to CDRs of a non-human antibody and all or substantially all of the framework regions (FRs) correspond to FRs of a human antibody. In certain such embodiments, a humanized antibody further comprises a constant region (Fc) of a human antibody.

The term "effective dose" or "effective amount" refers to an amount of an agent, e.g., an antibody, that results in the reduction of symptoms in a patient or results in a desired biological outcome. In certain embodiments, an effective dose or effective amount is sufficient to treat or reduce symptoms of a disease or condition.

DETAILED DESCRIPTION

Provided herein are methods of treatment and/or prevention of cancer by manipulation of commensal microflora. In particular, the amount, identity, presence, and/or ratio of microflora (e.g., gut microflora) in a subject is manipulated to facilitate one or more co-treatments.

T cell infiltration of solid tumors is associated with favorable patient outcomes, yet the mechanisms underlying variable endogenous immune responses between individuals are not well understood. Experiments were conducted during development of embodiments described herein to examine potential effects of microbial composition on spontaneous anti-tumor immunity. B16 melanoma growth was compared in C57BL/6 mice having distinct commensal microbiota. The two populations of mice showed robust versus weak spontaneous anti-tumor immunity. This phenotypic difference was eliminated upon cohousing or following fecal transfer. 16S rRNA sequencing identified *Bifidobacterium* as associated with the anti-tumor effects. Oral administration of *Bifidobacterium* alone or in combination with systemic αPD-L1 in tumor-bearing mice markedly improved tumor control in a $CD8^+$ T cell-dependent manner. Mechanistically, the effect was mediated by augmented dendritic cell function leading to more robust antigen-specific $CD8^+$ T cell priming and markedly increased accumulation of activated T cells in the tumor microenvironment. These data, for example, demonstrate advantages manipulating commensal microbes as a cancer therapeutic.

In some embodiments, the effectiveness of an endogenous immune response, immunotherapy, chemotherapeutic, or other treatment (e.g., surgery, radiation, etc.) in the treatment or prevention of reoccurrence of cancer and/or tumor is dependent upon conditions within the subject (e.g., the tumor microenvironment). In particular, the identity or characteristics (e.g., concentration or level) of the microflora within a subject affects the effectiveness of cancer treatments (e.g., generally or specific treatments) and/or the effectiveness of the subject's own immune response to cancer.

In some embodiments, the presence or increased level of one or more microbes (e.g., one or more types of bacteria) in a subject potentiates cancer/tumor growth, spread (e.g., malignancy), and/or evasion of treatment/immune response. In some embodiments, the presence or increased level of one or more microbes (e.g., one or more types of bacteria) in a subject inhibits treatment (e.g., immunotherapy, chemotherapy, etc.) and/or the subject's endogenous immune response to cancer and/or tumor cells. In some embodiments, the absence and/or decreased level of one or more microbes (e.g., one or more types of bacteria) in a subject potentiates cancer/tumor growth, spread, and/or evasion of treatment/immune response. In some embodiments, the absence or decreased level of one or more microbes (e.g., one or more types of bacteria) in a subject inhibits treatment (e.g., immunotherapy, chemotherapy, etc.) and/or the subject's endogenous immune response to cancer and/or tumor cells.

In some embodiments, the presence or increased level of one or more microbes (e.g., one or more types of bacteria) in a subject discourages cancer/tumor growth, spread, and/or evasion of treatment/immune response. In some embodiments, the presence or increased level of one or more microbes (e.g., one or more types of bacteria) in a subject facilitates treatment (e.g., immunotherapy, chemotherapy, etc.) and/or the subject's endogenous immune response to cancer and/or tumor cells. In some embodiments, the absence and/or decreased level of one or more microbes (e.g., one or more types of bacteria) in a subject discourages cancer/tumor growth, spread, and/or evasion of treatment/immune response. In some embodiments, the absence or decreased level of one or more microbes (e.g., one or more types of bacteria) in a subject facilitates treatment (e.g., immunotherapy, chemotherapy, etc.) and/or the subject's endogenous immune response to cancer and/or tumor cells.

In some embodiments, the presence of beneficial microbes (e.g., microbes that facilitate cancer treatment) in a subject creates an environment or microenvironment (e.g., metabolome) that is conducive to the treatment of cancer and/or inhibits cancer/tumor growth. In some embodiments, the presence of detrimental microbes (e.g., microbes that facilitate cancer/tumor growth and/or prevent treatment) in a subject creates an environment or microenvironment (e.g., metabolome) that is conducive to the treatment of cancer and/or inhibits cancer/tumor growth.

Experiments conducted during development of embodiments described herein demonstrate that modulation of levels and/or identity of the microflora in a subject facilitates treatment of cancer/tumor within the subject, enhances the endogenous immune response,

decreases immune evasion or other inhibitory mechanisms to treatment of endogenous immune response, and/or improves cancer outcomes for the subject. Modulation of microflora levels and/or identity may comprise encouraging or facilitating growth of one or more types of beneficial microbes (e.g., microbes that facilitate cancer treatment), discouraging or inhibiting growth of one or more types of detrimental microbes (e.g., microbes that facilitate cancer/tumor growth and/or prevent treatment), administering one or more types of beneficial microbes (e.g., microbes that facilitate cancer treatment) to the subject, and/or combinations thereof. Embodiments within the scope herein are not limited by the mechanisms for introducing one or more microbes (e.g., fecal transplant, probiotic administration, etc.), encouraging growth of beneficial microbes (e.g., administering agents that skew the environment within the subject toward growth conditions for the beneficial microbes), discouraging or inhibiting growth of detrimental microbes (e.g., administering agents that skew the environment within the subject away from growth conditions for the detrimental microbes, administration of antimicrobial(s), etc.), and combinations thereof.

In some embodiments, methods are provided for the treatment or prevention of cancer by the manipulation of the presence, amount, or relative ratio of commensal microflora (e.g., gut microflora). In some embodiments, the presence, amount, or relative ratio of particular bacteria, fungi, and/or archaea within a subject is manipulated. For example, in some embodiments, the presence, amount, or relative ratio of one or more bacteria from the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and/or *Proteobacteria* are manipulated. In some embodiments, the presence, amount, or relative ratio of one or more bacteria belonging to the genera *Bacteroides*, *Clostridium*, *Fusobacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Rikenella, Alistipes, Marinilabilia, Anaerostipes, Escherichia*, and/or *Lactobacillus* are manipulated. In some embodiments, the presence, amount, or relative ratio of one or more fungi belonging to the genus *Candida*, *Saccharomyces*, *Aspergillus*, and/or *Penicillium* are manipulated.

In some embodiments, the presence and/or levels of one or more commensal microbes are manipulated in a subject suffering from cancer, at heightened risk of cancer, and/or receiving treatment for cancer. Exemplary commensal microbes include *Lactococcus* (e.g., *Lactococcus cremoris* and *Lactococcus lactis*), *Lactobacillus* (e.g., *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus kefiri, Lactobacillus bifidus, Lactobacillus brevis, Lactobacillus helveticus,*

Lactobacillus paracasei , Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus curvatus, Lactobacillus bulgaricus, Lactobacillus sakei, Lactobacillus reuteri, Lactobacillus fermentum, Lactobacillus farciminis, Lactobacillus lactis, Lactobacillus delbrueckii, Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus johnsonii and *Lactobacillus jensenii*), *Leuconostoc, Carnobacterium, Enterococcus, Propionibacteium, Pediococcus, Bifidobacterium* (e.g., *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Bifidobacteriumangulatum*, etc.), Streptococcus (e.g., Stre*ptococcus thermophiles, Streptococcus salivarius, Streptococcus oralis, Streptococcus uberis, Streptococcus rattus*, etc.); *Escherichia coli, Bacillus coagulans, Bacillus lansii,* Yeast (e.g., Saccharomyces cerevisiae, Saccharomyces boulardii, etc.); and combinations thereof.

In some embodiments, one or more species, genera, and/or types of microbes are administered and/or the growth thereof is facilitated. In some embodiments, the growth of one or more species, genera, and/or types of microbes is inhibited. In some embodiments, one or more species, genera, and/or types of microbes are administered and/or the growth thereof is facilitated; and the growth of one or more other species, genera, and/or types of microbes is inhibited.

In some embodiments, the level or presence of one or more beneficial microbes (e.g., microbes that inhibit cancer/tumor growth or spread, enhance cancer/tumor treatment, etc.) is modulated by the administration of such microbes to a subject.

In some embodiments, microflora-modulation utilizes prepared probiotic compositions for administration to/by a subject. Probiotic compositions comprise one or more beneficial microbes (e.g., bacteria) formulated such that administration of the probiotic (e.g., orally, rectally, by inhalation, etc.) results in population of the subject by the beneficial microbes.

In some embodiments, probiotic compositions comprise cultured microbes that are combined and/or formulated for administration to a subject. In some embodiments, probiotics contain microbes of known genera, species, etc. and/or at known concentrations (cfus). Probiotic compositions may be in the form of a pharmaceutical-type composition (e.g., capsule, tables, liquid, aerosol, etc.) or in the form of a food supplement.

In some embodiments, probiotic microbes (e.g., bacteria) are formulated in a pharmaceutically acceptable composition for delivery to a subject. In some embodiments, probiotics are formulated with a pharmaceutically acceptable carrier suitable for a solid or semi-solid formulation. In some embodiments, probiotic microbes are formulated with a pharmaceutically acceptable carrier suitable for a liquid or gel formulation. Probiotic formulations may be formulated for enteral delivery, e.g., oral delivery, or delivery as a suppository, but can also be formulated for parenteral delivery, e.g., vaginal delivery, inhalational delivery (e.g., oral delivery, nasal delivery, and intrapulmonary delivery), and the like.

The probiotic compositions that find use in embodiments described herein may be formulated in a wide variety of oral administration dosage forms, with one or more pharmaceutically acceptable carriers. The pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances which may also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. In powders, the carrier is a finely divided solid which is a mixture with the probiotic microbes. In tablets, the microbes are mixed with the carrier having the necessary binding capacity in suitable proportions and compacted in the shape and size desired. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. Other forms suitable for oral administration include liquid form preparations such as emulsions, syrups, elixirs, aqueous solutions, aqueous suspensions, or solid form preparations which are intended to be converted shortly before use to liquid form preparations. Aqueous suspensions can be prepared by dispersing the probiotic microbes in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

The probiotic compositions (e.g., microbes (e.g., bacteria)) may be formulated for administration as suppositories. A low melting wax, such as a mixture of fatty acid glycerides or cocoa butter is first melted and the probiotic microbes are dispersed homogeneously, for

example, by stirring. The molten homogeneous mixture is then poured into conveniently sized molds, allowed to cool, and to solidify.

The probiotic compositions (e.g., microbes (e.g., bacteria)) may be formulated for vaginal administration. Pessaries, tampons, creams, gels, pastes, foams or sprays, may contain agents in addition to the bacteria, such carriers, known in the art to be appropriate.

In some embodiments, probiotic compositions (e.g., microbes (e.g., bacteria)) may be formulated for delivery by inhalation. As used herein, the term "aerosol" is used in its conventional sense as referring to very fine liquid or solid particles carries by a propellant gas under pressure to a site of therapeutic application. The term "liquid formulation for delivery to respiratory tissue" and the like, as used herein, describe compositions comprising probiotic microbes with a pharmaceutically acceptable carrier in flowable liquid form. Such formulations, when used for delivery to a respiratory tissue, are generally solutions, e.g. aqueous solutions, ethanolic solutions, aqueous/ethanolic solutions, saline solutions and colloidal suspensions.

Rather than pharmaceutical-type formulation, probiotic compositions may be formulated as food additive and/or food product and incorporated into a variety of foods and beverages. Suitable foods and beverages include, but are not limited to, yogurts, ice creams, cheeses, baked products such as bread, biscuits and cakes, dairy and dairy substitute foods, soy-based food products, grain-based food products, starch-based food products, confectionery products, edible oil compositions, spreads, breakfast cereals, infant formulas, juices, power drinks, and the like.

In some embodiments, a probiotic composition is administered over a dosing time period (e.g., < 1 minute, <1 hour, <2 hours, <4 hours, <6 hours, <12 hours, <24 hours, etc.) in an amount that is sufficient to provide a desired therapeutic benefit (e.g., as a single dose, in combination with other doses, in combination with a co-administered therapeutic, etc.) In some embodiments, the dose of the probiotic composition administered for the dosing time period is concentration of from about 10 to about 1 x 10^{14} colony forming units (cfu) of the commensal microbial agent(s) (e.g., 10 cfu, 100 cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, or any suitable ranges therein (e.g., from about 10^{2} cfu to about 10^{13} cfu, about 1 x 10^{4} to about 1 x 10^{11} cfu, about 1 x 10^{6} to about 1 x 10^{9} cfu, about 1 x 10^{10} to about 1 x 10^{12} cf, etc.), etc.).

In some embodiments, the microbial make-up of a probiotic composition consists or consists essentially of one or more beneficial microbes (e.g., bacteria). In some embodiments, the microbial make-up of a probiotic composition consists or consists essentially of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or any ranges therein (e.g., 1-4, 5-10, 8-20, etc.) strains and/or species of microbes. In some embodiments, fewer than 50 microbial strains (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, or any ranges therein (e.g., 1-4, 5-10, 8-20, etc.) are at least 50% (e.g., 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%. 99.9%, 99.99%) of the microbial population (e.g., by mass, by cfu, etc.) of a probiotic composition. For example, in some embodiments, a single species or strain of *Bifidobacterium* is at least 95% of the microbial population, as measured by colony forming units, of a particular probiotic composition. As another example, in some embodiments, a single species or strain of *Bifidobacterium* is at least 40% and bacteria from the genus *Lactobacillus* are at least 50% of the microbial population, as measured by mass, of a particular probiotic composition. These examples are not limiting.

In some embodiments, microflora in a subject (e.g., a subject suffering from cancer, a subject with microflora that promotes cancer growth, a subject with microflora that promotes evasion of cancer treatment (e.g., by immunotherapy), etc.) are modulated by transplantation of microbiota from a subject with favorable characteristics (e.g., a subject without cancer, a subject with microflora that inhibits cancer growth, a subject with microflora that promotes treatment of cancer (e.g., by immunotherapy), etc.) into the recipient subject.

In some embodiments, donor microflora are obtained sampling microflora from the desired region of the donor subject body (e.g., colon, oral cavity, vagina, etc.). In particular embodiments, fecal material (e.g., 100 g – 500 g) is obtained from a donor. The material may be administered to a recipient subject with or without subsequent preparation steps (e.g., diluting, mixing, oxygenating, filtering, supplementing (e.g., with prebiotics, with growth media, etc.), testing (e.g., for pathogens or detrimental microbes), etc.). The donor microflora (e.g., fecal material) may be administered without preservation (e.g., administered within 12 hours (e.g., <6 hours, <4 hours, <2 hours, <1 hour, etc.)) or may be preserved (e.g., frozen, freeze dried, etc.), for example, to allow for delay (e.g., 1 day, 2, days, 1 week, 1 month, or more) before delivery to the subject.

In some embodiments, donor microflora are proceed to remove one or more components. For example, parasitic of detrimental microbes may be removed or killed. Contaminants within the donor sample may be removed. In some embodiments, donor microflora is enriched for one or more specific microbes (e.g., 2-fold, 3-fold, 4 fold, 10-fold, 20-fold, or more enrichment). In some embodiments, donor microflora is enriched such that at least 1% of the microbes in the population are the desired beneficial microbes (e.g., 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more). In some embodiments, donor microflora are doped with one or more cultured beneficial microbes.

In particular embodiments, transplanted microflora may be administered to the recipient subject by any suitable delivery mechanism, including but not limited to enema, colonoscope, nasogastric or nasoduodenal tube, lavage or irrigation, or orally (e.g., in the form of a capsule).

In some embodiments, a commensal microbial agent or population of microbial agents is administered (e.g., via probiotic composition or microflora transplant) over a dosing time period (e.g., < 1 minute, <1 hour, <2 hours, <4 hours, <6 hours, <12 hours, <24 hours, etc.) in an amount that is sufficient to provide a desired therapeutic benefit (e.g., as a single dose, in combination with other doses, in combination with a co-administered therapeutic, etc.) In some embodiments, the dose of commensal microbial agent(s) administered for the dosing time period is concentration of from about 10 to about 1 x 10^{14} colony forming units (cfu) of the commensal microbial agent(s) (e.g., 10 cfu, 100 cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, 10^{13} cfu, or any suitable ranges therein (e.g., from about 10^{2} cfu to about 10^{13} cfu, about 1 x 10^{4} to about 1 x 10^{11} cfu, about 1 x 10^{6} to about 1 x 10^{9} cfu, about 1 x 10^{10} to about 1 x 10^{12} cf, etc.), etc.).

The dose can be administered in a single unit dose administered at any time during a day. Alternatively the loading dose can be administered in two or more doses administered at a single time of day or at two or more separate times of day.

Over the course of multiple dosing periods, the dose can be tapered from an initial dose to a higher dose (or increased from an initial dose to a higher dose), on predetermined timing or by the when the subject and/or clinician based on the results of the treatment. The appropriate dosage amount will vary by, for example, an individual subject's age, weight, condition or disease, severity of disease, etc.

By way of non-limiting example (both in terms of identify of the microbe as well as dose), in some embodiments, one or more strains of *Bifidobacterium* are administered via 3 capsules daily, each capsule containing 1 x 10^9 cfu of *Bifidobacterium*. Alternatively, in other embodiments one or more strains of *Bifidobacterium* are administered at a dosage of one capsule daily containing 1 x 10^{12} cfu of bacteria. Any other dosages (e.g., cfu), doses (e.g., times per day, week, etc.), and identity of the microbe(s) (e.g., within the ranges described herein) are within the scope herein.

In some embodiments, microbes for probiotic compositions are obtained from culture. In some embodiments, strains of beneficial microbes are genetically engineered to enhance one or more of production (e.g., at scale), formulation, delivery, or the biological effect of the microbe. In some embodiments, microbes are engineered to express a detectable marker that allows tracking of the microbes within a subject, or confirmation that the microbe has integrated into a subjects microflora. In some embodiments, microbes are engineered to express a cancer therapeutic (e.g., chemotherapeutic, immunotherapeutic, antibodies, etc.), anti-inflammatory agent, of other drug.

In some embodiments, one or more prebiotics are administered to a subject as an independent treatment (e.g., to increase the level of a beneficial microbe) or in conjunction with other treatments described herein. Prebiotics are agents that increase the in vivo growth rate or activity of commensal microbes, such as *Lactobacillus* and/or *Bifidobacterium*. In some embodiments, prebiotics are soluble fiber sources. In some embodiments, when prebiotics are administered (e.g., fed) to a subject they are not digested or are not fully digested by the subject's digestive enzymes, but rather support the intestinal health of the subject and provide an energy source for the beneficial microbes and enhance the growth thereof. Prebiotics include, for example, naturally occurring lecithins and/or oleic acid, and are described, for example in U.S. Pat. No. 8,449,878 which is herein incorporated by reference in its entirety.

In some embodiments, the level or presence of one or more detrimental microbes (e.g., microbes that facilitate cancer/tumor growth or spread, inhibit cancer/tumor treatment, etc.) is modulated, for example, by the administration of one or more antimicrobial agents to a subject or modulation of conditions within the subject to disfavor growth of the detrimental microbes. In some embodiments, antimicrobial agents are administered.

In some embodiments, the antimicrobial agent is an antibiotic. Exemplary antibiotics that may find use in some embodiments include, but are not limited to: amikacin, gentamicin, kanamycin, neomycin, netilmicin, streptomycin, tobramycin, paromycin, geldanamycin, herbimycin, loracarbef, ertapenem, doripenem, imipenem, meropenem, cefaclor, cefamandole, cefotoxin, cefprozil, cefuroxime, cefixime, cefdinir, cefditoren, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, cefepime, ceftobirprole, vancomycin, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, aztreonam, amoxicillin, ampicillin, azociling, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, meticillin, nafcillin, oxacillin, peperacillin, ticarcillin, bacitracin, colistin, polymyxin B, ciprofloxacin, clavulanic acid, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, nonfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, AL-15469A, AL-38905, OP-145, afenide, prontosil, sulfacetamide, sulfamethiazole, sulfanamide, sulfasalazine, sulfisoxazole, trimethoprim, cotrimoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetraycline, linezolid, arsogebanubem chloramphenicol, clindamycin, lincomycin, ethambutol, fosfomycin, fusidic acid, furazolidone, isoniazid, linezolid, metronidazole, mupirocin, nitrofurantoin, rifampicin, thamphenicol, tinidazole, amoxicillin+clavulanic acid, Maximin H5, Dermcidin, Cecropins, andropin, moricin, ceratotoxin, melittin, Magainin, dermaseptin, bombinin, brevinin-l,esculentins and buforin II, CAP 18, LL37 , abaecin, apidaecins, prophenin, indolicidin, brevinins, protegrin, tachyplesins, defensins, drosomycin, alamethicin, pexiganan or MSI-78, MSI-843, MSI-594, polyphemusin, colicin, pyocin, klebicin, subtilin, epidermin, herbicolacin, brevicin, halocin , agrocin, alveicin, carnocin, curvaticin, divercin ,enterocin, enterolysin, erwiniocin, glycinecin, lactococin, lacticin, leucoccin, mesentericin, pediocin, plantaricin, sakacin, sulfolobicin, vibriocin, warnerinand, nisin, or a salt or cocrystal, or prodrug or solvate thereof, or a combination thereof.

In some embodiments, the antimicrobial is an antifungal agent. Exemplary antifungals that may find use in some embodiments include, but are not limited to: amrolfine, utenafine, naftifine, terbinafine, flucytosine, fluconazole, itraconazole, ketoconazole, posaconazole, ravuconazole, voriconazole, clotrimazole, econazole, miconazole, oxiconazole, sulconazole, terconazole, tioconazole, nikkomycin Z, caspofungin, micafungin, anidulafungin, amphotericin

B, liposomal nystastin, pimaricin, griseofulvin, ciclopirox olamine, haloprogin, tolnaftate, undecylenate, clioquinol, and combinations thereof.

In some embodiments, the antimicrobial is an antiparasitic. Exemplary antiparasitics that may find use in some embodiments include, but are not limited to:amitraz, amoscanate, avermectin, carbadox, diethylcarbamizine, dimetridazole, diminazene, ivermectin, macrofilaricide, malathion, mitaban, oxamniquine, permethrin, praziquantel, prantel pamoate, selamectin, sodium stibogluconate, thiabendazole, and combinations thereof.

In some embodiments, methods and compositions for reduction of detrimental microbe levels are co-administered (e.g., serially, concurrently, etc.) with methods and compositions for increasing beneficial microbe levels. In some embodiments, by reducing overall microbe levels or by reducing the levels of specific microbes (e.g., detrimental microbes, high population microbes, etc.), the population of beneficial microbes can more effectively be modulated (e.g., increased).

In some embodiments, in order to develop a microflora population within a subject that facilitates cancer treatment or inhibits cancer growth/spread, antimicrobial agents are first administered to eliminate or reduce the microflora within the subject, and then the microflora population is reestablished using the methods and compositions described herein (e.g., administration of beneficial microbes). In some embodiments, antimicrobials (e.g., antibiotics) that reduce the microbe (e.g., bacteria) population generally are employed. In some embodiments, antimicrobials that target detrimental microbes preferentially are employed.

In some embodiments, modulating the microflora composition is sufficient on its own to allow the endogenous immune system of a subject to respond to the presence of cancer cells and or tumor growth. However, in other embodiments, microflora composition is manipulated along with one or more other cancer therapies. In some embodiments, manipulation of the microflora composition (e.g., identity and/or level) treats cancer by a mechanism independent of one or more additional cancer treatments. In other embodiments, modulation of microflora composition facilitates (e.g., increases the effectiveness of) the cancer treatment. In some embodiments, one or more cancer treatments enhance the effectiveness of the modulation of microflora composition. Embodiments herein are not limited by the types of cancer treatments (e.g., surgery, radiation, immunotherapy, chemotherapeutic, etc.) unless specifically noted.

In some embodiments, immunotherapeutic cancer treatment encompasses blockade of immune-inhibitory receptors, for example using monoclonal antibodies (mAbs) against CTLA-4 and PD-1/PD-L1 (Wolchok, J. D. et al. The New England Journal of Medicine 369, 122-133 (2013).; Topalian, S. L. et al. Journal of clinical oncology 32, 1020-1030 (2014).; Topalian, S. L. et al. The New England journal of medicine 366, 2443-2454 (2012).; Hodi, F. S. et al. The New England journal of medicine 363, 711-723 (2010).; herein incorporated by reference in their entireties).

In some embodiments, the immunotherapy includes the administration of an immune checkpoint inhibitor. Immune Checkpoint inhibition broadly refers to inhibiting the checkpoints that cancer cells can produce to prevent or downregulate an immune response. Examples of immune checkpoint proteins include, but are not limited to, CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA. Immune checkpoint inhibitors can be antibodies or antigen binding fragments thereof that bind to and inhibit an immune checkpoint protein. Examples of immune checkpoint inhibitors include, but are not limited to, nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT Ol l, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010. In some embodiments, the immune checkpoint inhibitor may be administered via injection (e.g., intravenously, intratumorally, subcutaneously, or into lymph nodes), but may also be administered orally, topically, or via aerosol.

In some embodiments, the compositions for and/or methods of modulating microflora in a subject overcome immune invasion of cancer cells, tumor, tumor microenvironment, etc. In some embodiments, one or more additional cancer immunotherapies are employed (e.g., concurrently or serially) to make use of the induced immune-responsiveness treated cells/tumor. Suitable immunotherapies may include, but are not limited to: cell-based therapies (e.g., dendritic cell or T cell therapy, etc.), monoclonal antibody (mAb) therapy (e.g., naked mAbs, conjugated mAbs), cytokine therapy (e.g., interferons, interleukins, etc.), adjuvant treatment (e.g., polysaccharide-K), etc.

Examples of antibodies that may find use in the compositions and methods disclosed herein, particularly for use in immunotherapies (but not so limited) include, but are not limited, to antibodies such as trastuzumab (anti-HER2/neu antibody); Pertuzumab (anti-HER2 mAb); cetuximab (chimeric monoclonal antibody to epidermal growth factor receptor EGFR);

panitumumab (anti-EGFR antibody); nimotuzumab (anti-EGFR antibody); Zalutumumab (anti-EGFR mAb); Necitumumab (anti-EGFR mAb); MDX-210 (humanized anti-HER-2 bispecific antibody); MDX-210 (humanized anti-HER-2 bispecific antibody); MDX-447 (humanized anti-EGF receptor bispecific antibody); Rituximab (chimeric murine/human anti-CD20 mAb); Obinutuzumab (anti-CD20 mAb); Ofatumumab (anti-CD20 mAb); Tositumumab-1131 (anti-CD20 mAb); Ibritumomab tiuxetan (anti-CD20 mAb); Bevacizumab (anti-VEGF mAb); Ramucirumab (anti-VEGFR2 mAb); Ranibizumab (anti-VEGF mAb); Aflibercept (extracellular domains of VEGFR1 and VEGFR2 fused to IgG1 Fc); AMG386 (angiopoietin-1 and -2 binding peptide fused to IgG1 Fc); Dalotuzumab (anti-IGF-1R mAb); Gemtuzumab ozogamicin (anti-CD33 mAb); Alemtuzumab (anti-Campath-1/CD52 mAb); Brentuximab vedotin (anti-CD30 mAb): Catumaxomab (bispecific mAb that targets epithelial cell adhesion molecule and CD3); Naptumomab (anti-5T4 mAb); Girentuximab (anti-Carbonic anhydrase ix); or Farletuzumab (anti-folate receptor). Other examples include antibodies such as Panorex™ (17-1A) (murine monoclonal antibody); Panorex (@(17-1A)) (chimeric murine monoclonal antibody); BEC2 (ami-idiotypic mAb, mimics the GD epitope) (with BCG); Oncolym (Lym-1 monoclonal antibody); SMART M195 Ab, humanized 13′ 1 LYM-1 (Oncolym). Ovarex (B43.13, anti-idiotypic mouse mAb); 3622W94 mAb that binds to EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas; Zenapax (SMART Anti-Tac (IL-2 receptor); SMART M195 Ab, humanized Ab, humanized); NovoMAb-G2 (pancarcinoma specific Ab); TNT (chimeric mAb to histone antigens); TNT (chimeric mAb to histone antigens); Gliomab-H (Monoclonals—Humanized Abs); GNI-250 Mab; EMD-72000 (chimeric-EGF antagonist); LymphoCide (humanized IL.L.2 antibody); and MDX-260 bispecific, targets GD-2, ANA Ab, SMART IDIO Ab, SMART ABL 364 Ab, or ImmuRAIT-CEA.

In some embodiments, an immunotherapy, utilized as a co-therapy with the microflora modulation described herein, directly or indirectly targets one of more of: a regulatory T cell, myeloid suppressor cell, or dendritic cell. In another aspect, an immunotherapy specifically targets one of the following molecules: CD4; CD25 (IL-2α receptor; IL-2αR); cytotoxic T-lymphocyte antigen-4 (CTLA-4; CD152); Interleukin-10 (IL-10); Transforming growth factor-beta receptor (TGF-βR); Transforming growth factor-beta (TGF-β); Programmed Death-1 (PD-1); Programmed death-1 ligand (PD-L1 or PD-L2); Receptor activator of nuclear factor-κB (RANK); Receptor activator of nuclear factor-κB (RANK) ligand (RANKL); LAG-3;

glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR; TNFRSF18); or Interleukin-4 receptor (IL-4R). In some embodiments, the immunotherapy acts as an agonist that increases the function of the targeted molecule. In other embodiments, the immunotherapy is an antagonist that inhibits the function of the targeted molecule.

In some embodiments, an immunotherapy, utilized as a co-therapy with the microflora modulation described herein, directly or indirectly targets one of more of a specific cytokine, cytokine receptor, co-stimulatory molecule, co-inhibitory molecule, or immunomodulatory receptor that modulates the immune system. In another aspect, one of the following molecules are targeted by co-treatment with microflora modulation: tumor necrosis factor (TNF) superfamily; tumor necrosis factor-α (TNF-α); tumor necrosis factor receptor (TNFR) superfamily; Interleukin-12 (IL-12); IL-12 receptor; 4-1BB (CD137); 4-1BB ligand (4-1BBL; CD137L); OX40 (CD134; TNR4); OX40 ligand (OX40L; CD40; CD40 ligand (CD40L); CTLA-4; Programmed death-1 (PD-1); PD-1 ligand I (PD-L1: B7-H1); or PD-1 ligand 2 (PD-L2; B7-DC); B7 family; B7-1 (CD80); B7-2 (CD86); B7-H3; B7-H4; GITR/AITR: GITRL/AITRL; BTLA; CD70; CD27; LIGHT; HVEM: Toll-like receptor (TLR) (TLR 1, 2, 3, 4, 5, 6, 7, 8, 9, 10).

In some embodiments, the compositions for and/or methods of modulating microflora in a subject sensitize the cancer cells and/or tumor to treatment by one or more chemotherapeutic agents. In some embodiments, one or more chemotherapies are employed in addition to microflora modulation (e.g., concurrently or serially) to make use of the induced chemotherapeutic sensitivity. In other embodiments, one or more chemotherapeutics are provided as co-therapies with microflora modulation, with or without (known) synergism between the microflora modulation and the chemotherapy.

In some embodiments, exemplary anticancer agents suitable for use in compositions and methods described herein (e.g., co-administered with a β-catenin inhibitor) include, but are not limited to: 1) alkaloids, including microtubule inhibitors (e.g., vincristine, vinblastine, and vindesine, etc.), microtubule stabilizers (e.g., paclitaxel (Taxol), and docetaxel, etc.), and chromatin function inhibitors, including topoisomerase inhibitors, such as epipodophyllotoxins (e.g., etoposide (VP-16), and teniposide (VM-26), etc.), and agents that target topoisomerase I (e.g., camptothecin and isirinotecan (CPT-11), etc.); 2) covalent DNA-binding agents (alkylating agents), including nitrogen mustards (e.g., mechlorethamine, chlorambucil, cyclophosphamide,

ifosphamide, and busulfan (MYLERAN), etc.), nitrosoureas (e.g., carmustine, lomustine, and semustine, etc.), and other alkylating agents (e.g., dacarbazine, hydroxymethylmelamine, thiotepa, and mitomycin, etc.); 3) noncovalent DNA-binding agents (antitumor antibiotics), including nucleic acid inhibitors (e.g., dactinomycin (actinomycin D), etc.), anthracyclines (e.g., daunorubicin (daunomycin, and cerubidine), doxorubicin (adriamycin), and idarubicin (idamycin), etc.), anthracenediones (e.g., anthracycline analogues, such as mitoxantrone, etc.), bleomycins (BLENOXANE), etc., and plicamycin (mithramycin), etc.; 4) antimetabolites, including antifolates (e.g., methotrexate, FOLEX, and MEXATE, etc.), purine antimetabolites (e.g., 6-mercaptopurine (6-MP, PURINETHOL), 6-thioguanine (6-TG), azathioprine, acyclovir, ganciclovir, chlorodeoxyadenosine, 2-chlorodeoxyadenosine (CdA), and 2'-deoxycoformycin (pentostatin), etc.), pyrimidine antagonists (e.g., fluoropyrimidines (e.g., 5-fluorouracil (ADRUCIL), 5-fluorodeoxyuridine (FdUrd) (floxuridine)) etc.), and cytosine arabinosides (e.g., CYTOSAR (ara-C) and fludarabine, etc.); 5) enzymes, including L-asparaginase, and hydroxyurea, etc.; 6) hormones, including glucocorticoids, antiestrogens (e.g., tamoxifen, etc.), nonsteroidal antiandrogens (e.g., flutamide, etc.), and aromatase inhibitors (e.g., anastrozole (ARIMIDEX), etc.); 7) platinum compounds (e.g., cisplatin and carboplatin, etc.); 8) monoclonal antibodies (e.g., conjugated with anticancer drugs, toxins, and/or radionuclides, etc.; neutralizing antibodies; etc.); 9) biological response modifiers (e.g., interferons (e.g., IFN-.alpha., etc.) and interleukins (e.g., IL-2, etc.), etc.); 10) adoptive immunotherapy; 11) hematopoietic growth factors; 12) agents that induce tumor cell differentiation (e.g., all-trans-retinoic acid, etc.); 13) gene therapy techniques; 14) antisense therapy techniques; 15) tumor vaccines; 16) therapies directed against tumor metastases (e.g., batimastat, etc.); 17) angiogenesis inhibitors; 18) proteosome inhibitors (e.g., VELCADE); 19) inhibitors of acetylation and/or methylation (e.g., HDAC inhibitors); 20) modulators of NF kappa B; 21) inhibitors of cell cycle regulation (e.g., CDK inhibitors); and 22) modulators of p53 protein function.

In some embodiments, compositions and methods herein comprise multiple modes for the treatment and/or prevention of cancer. In some embodiments, beneficial microbes are provided/administered (e.g., by a probiotic composition, fecal transplant, etc.) with prebiotics and/or other agents that facilitate the growth of the beneficial microbes. In some embodiments, beneficial microbes are provided/administered (e.g., by a probiotic composition, fecal transplant, etc.) with antimicrobial(s) (e.g., antibiotics) directed to kill or inhibit the growth of detrimental

microbes. In some embodiments, prebiotics and/or other agents that facilitate the growth of the beneficial microbes are provided/administered with antimicrobial(s) (e.g., antibiotics) directed to kill or inhibit the growth of detrimental microbes. In some embodiments, beneficial microbes, prebiotics and/or other agents that facilitate the growth of the beneficial microbes, and an antimicrobial(s) (e.g., antibiotics) directed to kill or inhibit the growth of detrimental microbes are all co-administered.

In some embodiments, the co-administered agents are formulated into a single dose and/or composition. In some embodiments, the co-administered agents are in separate doses and/or compositions. In some embodiments in which separate doses and/or compositions are administered, the doses and/or compositions are administered simultaneously, consecutively, or spaced over a time span (e.g., <30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, or more, or any suitable ranges therebetween).

In some embodiments, beneficial microbes, prebiotics and/or other agents that facilitate the growth of the beneficial microbes, antimicrobial(s) (e.g., antibiotics) directed to kill or inhibit the growth of detrimental microbes, or any of the above mentioned combinations thereof are administered with a treatment for cancer. In embodiments, in which the modulation of microflora itself provides treatment for cancer, suitable co-treatments include immunotherapy, chemotherapy, surgery (e.g., tumor removal), radiation, etc. In other embodiments, in which the modulation of microflora sensitizes a subject or the tumor microenvironment to a particular cancer therapy (e.g., an immunotherapy, a chemotherapy, etc.), the particular cancer therapy is administered (e.g., optionally in addition to one or more other cancer therapies to which the subject is not directly sensitized to by the modulation).

In some embodiments, microflora modulation is provided as a co-therapy (e.g., chemotherapy, immunotherapy, etc.) with one or more additional therapies that target and/or bind to specific cancer or tumor cell markers. Such markers may be selected from the group including but not limited to, epidermal growth factor receptor (EGFR, EGFR1, ErbB-1, HER1). ErbB-2 (HER2/neu), ErbB-3/HER3, ErbB-4/HER4, EGFR ligand family; insulin-like growth factor receptor (IGFR) family, IGF-binding proteins (IGFBPs), IGFR ligand family (IGF-1R); platelet derived growth factor receptor (PDGFR) family, PDGFR ligand family; fibroblast growth factor receptor (FGFR) family, FGFR ligand family, vascular endothelial growth factor receptor (VEGFR) family, VEGF family; HGF receptor family: TRK receptor family; ephrin

(EPH) receptor family: AXL receptor family; leukocyte tyrosine kinase (LTK) receptor family; TIE receptor family, angiopoietin 1, 2; receptor tyrosine kinase-like orphan receptor (ROR) receptor family; discoidin domain receptor (DDR) family; RET receptor family; KLG receptor family; RYK receptor family; MuSK receptor family; Transforming growth factor alpha (TGF-α), TGF-α receptor; Transforming growth factor-beta (TGF-β), TGF-β receptor; Interleukin β receptor alpha2 chain (IL13Ralpha2), Interleukin-6 (IL-6), 1L-6 receptor, interleukin-4, IL-4 receptor, Cytokine receptors, Class I (hematopoietin family) and Class II (interferon/1L-10 family) receptors, tumor necrosis factor (TNF) family, TNF-α, tumor necrosis factor (TNF) receptor superfamily (TNTRSF), death receptor family, TRAIL-receptor; cancer-testis (CT) antigens, lineage-specific antigens, differentiation antigens, alpha-actinin-4, ARTC1, breakpoint cluster region-Abelson (Bcr-abl) fusion products, B-RAF, caspase-5 (CASP-5), caspase-8 (CASP-8), beta-catenin (CTNNB1), cell division cycle 27 (CDC27), cyclin-dependent kinase 4 (CDK4), CDKN2A, COA-1, dek-can fusion protein, EFTUD-2, Elongation factor 2 (ELF2), Ets variant gene 6/acute myeloid leukemia 1 gene ETS (ETC6-AML1) fusion protein, fibronectin (FN), GPNMB, low density lipid receptor/GDP-L fucose: beta-Dgalactose 2-alpha-Lfucosyltraosferase (LDLR/FUT) fusion protein, HLA-A2, MLA-A11, heat shock protein 70-2 mutated (HSP70-2M), KIAA0205, MART2, melanoma ubiquitous mutated 1, 2, 3 (MUM-1, 2, 3), prostatic acid phosphatase (PAP), neo-PAP, Myosin class 1, NFYC, OGT, OS-9, pml-RARalpha fusion protein, PRDX5, PTPRK, K-ras (KRAS2), N-ras (NRAS), HRAS, RBAF600, SIRT12, SNRPD1, SYT-SSX1 or -SSX2 fusion protein, Triosephosphate Isomerase, BAGE, BAGE-1, BAGE-2, 3, 4, 5, GAGE-1, 2, 3, 4, 5, 6, 7, 8, GnT-V (aberrant N-acetyl glucosaminyl transferase V, MGAT5), HERV-K MEL, KK-LC, KM-HN-1, LAGE, LAGE-1, CTL-recognized antigen on melanoma (CAMEL), MAGE-A1 (MAGE-1). MAGE-A2, MAGE-A3, MAGE-A4, MAGE-AS, MAGE-A6, MAGE-A8, MAGE-A9, MAGE-A10. MAGE-All, MAGE-A12, MAGE-3, MAGE-B1, MAGE-B2, MAGE-B5. MAGE-B6, MAGE-C1, MAGE-C2, mucin 1 (MUC1), MART-1/Melan-A (MLANA), gp100, gp100/Pme117 (S1LV), tyrosinase (TYR), TRP-1, HAGE, NA-88, NY-ESO-1, NY-ESO-1/LAGE-2, SAGE, Sp17. SSX-1, 2, 3, 4, TRP2-1NT2, carcino-embryonic antigen (CEA), Kallikrein 4, mammaglobin-A, OA1, prostate specific antigen (PSA), prostate specific membrane antigen, TRP-1/, 75. TRP-2 adipophilin, interferon inducible protein absent in melanorna 2 (AIM-2). BING-4, CPSF, cyclin D1, epithelial cell adhesion molecule (Ep-CAM), EpbA3, fibroblast growth factor-5 (FGF-5), glycoprotein 250

(gp250intestinal carboxyl esterase (iCE), alpha-feto protein (AFP), M-CSF, mdm-2, MUCI, p53 (TP53), PBF, PRAME, PSMA, RAGE-1, RNF43, RU2AS, SOX10, STEAP1, survivin (BIRCS), human telomerase reverse transcriptase (hTERT), telomerase, Wilms' tumor gene (WT1), SYCP1, BRDT, SPANX, XAGE, ADAM2, PAGE-5, LIP1, CTAGE-1, CSAGE, MMA1, CAGE, BORIS, HOM-TES-85, AF15q14, HCA66I, LDHC, MORC, SGY-1, SPO11, TPX1, NY-SAR-35, FTHLI7, NXF2 TDRD1, TEX 15, FATE, TPTE, immunoglobulin idiotypes, Bence-Jones protein, estrogen receptors (ER), androgen receptors (AR), CD40, CD30, CD20, CD19, CD33, CD4, CD25, CD3, cancer antigen 72-4 (CA 72-4), cancer antigen 15-3 (CA 15-3), cancer antigen 27-29 (CA 27-29), cancer antigen 125 (CA 125), cancer antigen 19-9 (CA 19-9), beta-human chorionic gonadotropin, 1-2 microglobulin, squamous cell carcinoma antigen, neuron-specific enolase, heat shock protein gp96. GM2, sargramostim, CTLA-4, 707 alanine proline (707-AP), adenocarcinoma antigen recognized by T cells 4 (ART-4), carcinoembryogenic antigen peptide-1 (CAP-1), calcium-activated chloride channel-2 (CLCA2), cyclophilin B (Cyp-B), human signet ring tumor-2 (HST-2), etc.

Non-limiting examples of cancers that may be treated with the compositions and methods described herein include, but are not limited to: cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous

adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and roblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma

cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia. In some embodiments, the cancer is a melanoma (e.g., metastatic malignant melanoma), renal cancer (e.g. clear cell carcinoma), prostate cancer (e.g. hormone refractory prostate adenocarcinoma), pancreatic cancer (e.g., adenocarcinoma), breast cancer, colon cancer, gallbladder cancer, lung cancer (e.g. non-small cell lung cancer), esophageal cancer, squamous cell carcinoma of the head and neck, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, lymphoma, and other neoplastic malignancies. In some embodiments, the cancer is a solid tumor cancer.

In some embodiments, the methods provided herein relate to the treatment and/or prevention of a leukemia. The term "leukemia" is meant broadly progressive, malignant diseases of the hematopoietic organs/systems and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Non-limiting examples of leukemia diseases include, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemic leukemia, basophilic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, undifferentiated cell leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, and promyelocytic leukemia.

In some embodiments, the methods provided herein relate to the treatment and/or prevention of a carcinoma. The term "carcinoma" refers to a malignant growth made up of epithelial cells tending to infiltrate the surrounding tissues, and/or resist physiological and non-physiological cell death signals and gives rise to metastases. Non-limiting exemplary types of carcinomas include, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic

carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiennoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, carcinoma villosum, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypernephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, naspharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, and carcinoma scroti.

In some embodiments, the methods provided herein relate to the treatment and/or prevention of a sarcoma. The term "sarcoma" generally refers to a tumor which is made up of a substance like the embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar, heterogeneous, or homogeneous substance. Sarcomas include, but are not limited to, chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, endometrial sarcoma, stromal sarcoma, Ewing' s sarcoma, fascial sarcoma,

fibroblastic sarcoma, giant cell sarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms' tumor sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectaltic sarcoma.

Additional exemplary neoplasias that can be treated and/or prevented using the methods described herein include Hodgkin's Disease, Non-Hodgkin's Lymphoma, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insulanoma, malignant carcinoid, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, and adrenal cortical cancer.

In some embodiments, the cancer treated and/or prevented is a melanoma. The term "melanoma" is taken to mean a tumor arising from the melanocytic system of the skin and other organs. Non-limiting examples of melanomas are Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, nodular melanoma subungal melanoma, and superficial spreading melanoma.

Particular categories of tumors that can be treated and/or prevented using methods described herein include lymphoproliferative disorders, breast cancer, ovarian cancer, prostate cancer, cervical cancer, endometrial cancer, bone cancer, liver cancer, stomach cancer, colon cancer, pancreatic cancer, cancer of the thyroid, head and neck cancer, cancer of the central nervous system, cancer of the peripheral nervous system, skin cancer, kidney cancer, as well as metastases of all the above. Particular types of tumors include hepatocellular carcinoma, hepatoma, hepatoblastoma, rhabdomyosarcoma, esophageal carcinoma, thyroid carcinoma, ganglioblastoma, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, Ewing's tumor, leimyosarcoma,

rhabdotheliosarcoma, invasive ductal carcinoma, papillary adenocarcinoma, melanoma, pulmonary squamous cell carcinoma, basal cell carcinoma, adenocarcinoma (well differentiated, moderately differentiated, poorly differentiated or undifferentiated), bronchioloalveolar carcinoma, renal cell carcinoma, hypernephroma, hypernephroid adenocarcinoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, lung carcinoma including small cell, non-small and large cell lung carcinoma, bladder carcinoma, glioma, astrocyoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, retinoblastoma, neuroblastoma, colon carcinoma, rectal carcinoma, hematopoietic malignancies including all types of leukemia and lymphoma including: acute myelogenous leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, mast cell leukemia, multiple myeloma, myeloid lymphoma, Hodgkin' s lymphoma, non-Hodgkin' s lymphoma.

Cancers prevented and/or treated in certain embodiments also include precancerous lesions, e.g. actinic keratosis (solar keratosis), moles (dysplastic nevi), acitinic chelitis (farmer's lip), cutaneous horns, Barrett's esophagus, atrophic gastritis, dyskeratosis congenita, sideropenic dysphagia, lichen planus, oral submucous fibrosis, actinic (solar) elastosis and cervical dysplasia.

Cancers prevented and/or treated in some embodimentsinclude non-cancerous or benign tumors, e.g. of endodermal, ectodermal or mesenchymal origin, including, but not limited to cholangioma, colonic polyp, adenoma, papilloma, cystadenoma, liver cell adenoma, hydatidiform mole, renal tubular adenoma, squamous cell papilloma, gastric polyp, hemangioma, osteoma, chondroma, lipoma, fibroma, lymphangioma, leiomyoma, rhabdomyoma, astrocytoma, nevus, meningioma, and ganglioneuroma.

Some embodiments described herein are particularly useful for the treatment of tumors that do not otherwise respond to immunotherapeutic approaches. In some embodiments, such tumors are non-responsive (or have a reduced response) to T cells (e.g., prevent infiltration of one or more T cell types (e.g., $CD8^+$ T cells) or antigen presenting cells (e.g., dendritic cells (e.g., $CD103^+$DCs, etc.), etc.). In some embodiments, compositions and methods described herein find use in the treatment of cancers in which T cells are not appropriately primed against tumor-associated antigens.

In some embodiments, methods are provided for testing sample (e.g., cell, tissue, population of cells, tumor, blood, urine, saliva, etc.) from a subject for one or more biomarkers

of cancer, immune evasion, cancer promoting microenvironment, malignancy-promoting microenvironment, etc. Such biomarkers may comprise nucleic acids, small molecules, proteins, peptides, etc., and may be detected using any suitable assay of technique. In some embodiments, provided herein are DNA-, RNA-, small molecule, and/or protein-based diagnostic methods that either directly or indirectly detect the biomarkers of the evasion of immune response or immunotherapy by cancer cells or tumors. The present invention also provides compositions, reagents, and kits for such diagnostic purposes.

In some embodiments, biomarkers are detected at the nucleic acid (e.g., RNA) level. For example, the presence or amount of biomarker nucleic acid (e.g.,mRNA) in a sample is determined (e.g., to determine the presence or level of biomarker expression). Biomarker nucleic acid (e.g., RNA, amplified cDNA, etc.) may be detected/quantified using a variety of nucleic acid techniques known to those of ordinary skill in the art, including but not limited to nucleic acid sequencing, nucleic acid hybridization, nucleic acid amplification (e.g., by PCR, RT-PCR, qPCR, etc.), micorarray, Southern and Northern blotting, sequencing, etc. Non-amplified or amplified nucleic acids can be detected by any conventional means. For example, in some embodiments, nucleic acids are detected by hybridization with a detectably labeled probe and measurement of the resulting hybrids. Nucleic acid detection reagents may be labeled (e.g., fluorescently) or unlabeled, and may by free in solution or immobilized (e.g., on a bead, well, surface, chip, etc.).

In some embodiments, biomarkers are detected at the protein level. For example, the presence or amount of biomarker protein in a sample is determined (e.g., to determine the presence or level of biomarker expression or localization). In some embodiments, reagents are provided for the detection and/or quantification of biomarker proteins. Suitable reagents include primary antibodies (e.g., that bind to the biomarkers), secondary antibodies (e.g., that bind primary antibodies), antibody fragments, aptamers, etc. Protein detection reagents may be labeled (e.g., fluorescently) or unlabeled, and may by free in solution or immobilized (e.g., on a bead, well, surface, chip, etc.).

In some embodiments, kits are provided comprising, for example, the probiotic or microflora transplant compositions described herein. Kits may further comprise instructions, cancer treatments, other probiotics, agents to enhance integration of microbes into the subject's microflora, etc.

EXPERIMENTAL

Example 1

Materials and methods:

Animals and tumor model: C57BL/6 mice were obtained from Jackson laboratory or Taconic farms. 6–8-week-old female mice were used. The C57BL/6-derived melanoma cell line B16.F10.SIY (referred to herein as B16.SIY) was generated (Blank et al. Cancer research 64, 1140-1145 (2004).; herein incorporated by reference in its entirety). For tumor growth experiments, mice were injected subcutaneously with 1×10^6 B16.SIY tumor cells. Tumor size was measured twice a week until endpoint and tumor volume was determined as length × width^2 × 0.5. For B16 parental tumor model experiments, mice were injected subcutaneously with 1×10^6 B16.F10 tumor cells. For bladder cancer model experiments, mice were injected subcutaneously with $2x10^6$ MB49 cells. All experimental animal procedures were approved by the University of Chicago Animal Care and Use Committee (IACUC).

IFN-γ ELISPOT and SIY Pentamer analyses: Elispot plates (*Millipore, MAIP S4510*) were coated with purified αIFN-γ (*BD*) overnight at 4 °C. Plates were blocked with 10%FBS in DMEM for 2 hours at room temperature. Whole splenocytes were plated at 10^6 cells per well and stimulated with SIY peptide overnight at 37°C. Spots were developed using the BD mouse IFN-γ kit (*Cat. No. 552569*), and the number of spots was measured using an Immunospot Series 3 Analyzer and analyzed using ImmunoSpot software (Cellular Technology). For pentamer staining, cells were labeled with PE-MHC class I pentamer (*Proimmune*) consisting of murine H-2K^b complexed to SIYRYYGL (SIY) peptide or to control SIINFEKL peptide, and stained with CD3-AX700 (*Ebioscience, 17A2*), CD8-PacBlue (*Biolegend, 53-6.7*), CD4-APC (*Pharmingen, RM4-5*), CD62L-PECy7 (*Ebioscience, MEL-14*), CD44-FITC (*BD, IM7*) and Fixable Viability-ef780 (*Ebioscience*). Stained cells were analyzed using an LSR II cytometer with FACSDiva software (BD). Data analysis was conducted with FlowJo software (Tree Star).

Fecal transfers and αPD-L1 mAb immunotherapy: Fecal pellets from JAX and TAC-derived mice were collected upon arrival in our facility and each fecal pellet was resuspended in 1 ml of phosphate-buffered saline (PBS). The supernatant from each fecal pellet was used for oral gavage of two recipient mice, 100μl per gavage. For prophylactic fecal transfer experiments

mice were gavaged with JAX or TAC fecal suspensions once a week for two weeks prior to tumor inoculation. For therapeutic fecal transfer experiments, mice were gavaged on days 7 and 14 post tumor implantation. For combination therapy experiments, mice were additionally injected intraperitoneally with 100μg αPD-L1 mAb (*BioXCell*) in 100μl PBS on days 7, 10, 13 and 16 post-tumor implantation.

Microbial DNA analysis: Bacterial DNA was extracted from murine fecal pellets using PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio cat.# 12955-4). The V4-V5 region of the 16S rRNA encoding gene was amplified (earthmicrobiome.org/emp-standard-protocols/; Earth Microbiome Project, 2011) and sequenced at the High-Throughput Genome Analysis Core at Argonne National Laboratory. Quantitative Insights Into Microbial Ecology (QIIME) was used to trim and classify sequences (Caporaso et al. Bioinformatics 26, 266-267 (2010).; herein incorporated by reference in its entirety); specifically, the open reference OTU picking protocol was used at 97% sequence identity against the Greengenes database (05/13 release)(McDonald et al. The ISME journal 6, 610-618 (2012).;herein incorporated by reference in its entirety). PYNAST was used to align sequences (Caporaso et al. Nat Meth 7, 335-336 (2010).; herein incorporated by reference in its entirety) and RDP Classifier was used for taxonomic assignment (Wang et al. Appl Environ Microbiol 73, 5261-5267 (2007).; herein incorporated by reference in its entirety). Community structure was compared using weighted and unweighted UniFrac distances (Lozupone et al. Appl Environ Microbiol 71, 8228-8235 (2005).; herein incorporated by reference in its entirety). G-test was performed to determine differences in bacterial taxa occurrence between fecal communities. Prinicipal Coordinate Analysis (PCoA) ordination were generated to visually compare beta diversity and Analysis of Similarity (ANOSIM) test statistics were performed to statistically compare within- to between-group similarity in QIIME.

Bacterial administration and heat inactivation: A cocktail of lyophilized *Bifidobacterium* species (*B. bifidum, B. longum, B. lactis and B. breve*, Seeking Health) were resuspended in PBS at $5x10^9$ CFU/ml. Each mouse was given 200μl of *Bifidobacterium* ($1x10^9$ CFU/mouse) by oral gavage 7 and 14 days following tumor inoculation. Heat inactivation was performed by boiling rehydrated bifidobacteria at 100°C for 2 hours. Heat-treated and live bifidobacteria were serially diluted in reduced PBS and plated on reduced clostridial medium (RCM) agar in anaerobic conditions. Plates were subsequently incubated in an anaerobic chamber for three days to test

efficacy of killing. *Lactobacillus murinus* was cultured in MRS broth overnight, then washed and resuspended in PBS at $5x10^{10}$ CFU/ml. Each mouse was orally gavaged with 100µl of bacterial suspension ($5x10^9$ CFU/mouse) 7 and 14 days following tumor inoculation.

CFSE-labeled 2C CD8$^+$ T cell adoptive transfer: CD8$^+$ T cells were isolated from the spleen and lymph node of naïve CD45.1/.2$^+$ 2C TCR Tg mice using the MACS CD8 T cell Isolation Kit (*Miltenyi, Cat No. 130-095-236*), labeled with 2.5 mM CFSE and injected i.v. into CD45.2$^+$ C57BL/6 mice derived from either JAX or TAC. 24 hours later, mice were inoculated with $1x10^6$ B16.SIY melanoma cells s.c. Seven days post-adoptive T cell transfer, spleen and tumor-draining lymph node were harvested and restimulated ex-vivo with SIY peptide in the presence of brefeldin A. Samples were stained with Fixable Viability-ef780 (*Ebioscience*), CD45.1-PerCPCy5.5 (*Ebioscience, E20*), CD45.2-APC (*Ebioscience, 104*), CD3-AX700 (*Ebioscience, 17A2*), CD8-BV711 (*Biolegend, 53-6.7*), CD4-BV605 (*Biolegend, RM4-5*) and IFN-γ-PE (*BD, XMG1.2*). Intracellular IFN-γ production and CFSE dilution were assessed in gated CD45.1/.2$^+$ 2C CD8$^+$ T cells by flow cytometry.

Dendritic cell sorting and gene expression profiling: TAC mice were gavaged with *Bifidobacterium* once a week for two weeks. *Bifidobacterium*-fed mice, newly arrived JAX mice, and newly arrived TAC mice were inoculated subcutaneously in both flanks with $5x10^6$ DRAQ5-labeled B16.SIY tumor cells. 40hrs following tumor implantation, whole tumors including infiltrating immune cells were digested in collagenase (*Worthington*) and filtered into single cell suspensions. Samples from 5 mice in each group were pooled and subsequently stained with Fixable Viability-ef506 (*Ebioscience*), CD45-AF488 (*Bioloegend, 30-F11*), CD3-ef450 (*Ebioscience, 145-2C11*), CD19-PB (*Ebioscience, 1D3*), I-A/I-E-PECy7 (*Biolegend, M5/114.15.2*), CD11c-PE (*Ebioscience, N418*) and CD11b-PerCpCy5.5 (*BD, M1/70*). Live CD45$^+$CD3$^-$CD19$^-$MHCII^{hi}CD11c$^+$ dendritic cells were sorted directly into RLT Buffer (*Qiagen*) using FACSAriaIII (*BD*) and stored immediately on dry ice. Total RNA was isolated using RNeasy® Micro kit (*Qiagen*). RNA was submitted to the Functional Genomics Facility at the University of Chicago for gene expression profiling. RNA integrity and concentration were assessed using an Agilent Bioanalyzer 2100, and all RNA samples used for microarray analysis had an RNA Integrity Number > 9.0. Total RNA was processed into biotinylated cRNA using the Epicentre TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 (*TAB2R71024*). The

cRNA was hybridized to Illumina MouseRef8v2 arrays using Illumina provided protocols and scanned using an Illumina HiScan. Quantile normalized and background subtracted values were subsequently analyzed using R. Genes whose expression value was under 10 were removed from the analysis. Mean fold-change in gene transcript levels between JAX samples relative to TAC, and BIF samples relative to TAC were calculated, and genes whose fold change was over 1.5 in both comparisons (761 gene transcripts) were inputted into *The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**)* v6.7 for pathway analysis. Genes found to be significantly enriched ($p<0.05$) for immune function were then plotted in a heatmap using R software.

Statistical analysis: Tumor growth curves were analyzed using two-way ANOVA, with either Sidak's multiple comparisons posttest for comparison of two groups, or Tukey's multiple comparisons posttest for comparison of more than two groups. For comparisons other than tumor growth, Mann Whitney's non-parametric T-test was used when comparing two groups and one-way ANOVA with Tukey's multiple comparisons posttest was used when comparing more than two groups. $P<0.05$ was considered statistically significant and denoted as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Statistical analysis was performed using GraphPad PRISM.

Example 2

Results

Experiments were conducted during development of embodiments of the present invention to test whether differences in the specific composition of the normal microbiota influence the immune response to a growing tumor in vivo. Subcutaneous B16.SIY melanoma growth was observed in genetically similar C57BL/6 mice derived from two different mouse facilities, Jackson Laboratory (JAX) and Taconic Farms (TAC), which have been shown to differ in their commensal microbes (Ivanov et al. Cell 139, 485-498 (2009).; herein incorporated by reference in its entirety). It was found that JAX and TAC mice exhibited significant differences in B16.SIY melanoma growth rate, with tumors growing more aggressively in TAC mice (Figure 1A). To evaluate whether this difference was immune-mediated, tumor antigen-specific T cell responses, as well as T cell accumulation in the tumor microenvironment, were assessed. In fact, tumor-specific T cell responses were significantly higher in JAX mice (Figure 1B and 1C), and

markedly increased numbers of tumor-infiltrating T cells were observed (Figure 1D). To begin to address whether this difference could be mediated by commensal microbiota, JAX and TAC mice were co-housed for 3 weeks prior to tumor implantation. It was found that co-housing ablated the differences in tumor growth (Figure 1E) and immune responses (Figure 1F-H) between the two mouse populations, arguing for an environmental influence. Notably, TAC mice appeared to acquire the JAX phenotype upon cohousing, indicating that JAX mice might be colonized by commensal microbes that dominantly facilitate improved anti-tumor immunity.

To directly test the role of commensal bacteria in regulating anti-tumor immunity, JAX fecal suspensions or control TAC fecal suspensions were transferred into TAC recipients by oral gavage prior to tumor implantation Figure 5A). Strikingly, it was found that prophylactic transfer of JAX fecal material into TAC recipients was sufficient to delay tumor growth (Figure 2A) and enhance induction and infiltration of tumor-specific $CD8^+$ T cells (Figure 2B-C and 5B), supporting a microbe- or microbial product-derived effect. Reciprocal transfer of TAC fecal material into JAX recipients resulted in only a minimal increase in tumor growth rate and did not significantly alter anti-tumor T cell responses (Figure 2A-C and Figure 5B).

To test whether manipulation of the microbial community could be effective as a therapy, we administered JAX fecal material alone or in combination with antibodies targeting PD-L1 (αPD-L1) to TAC mice bearing established tumors. Transfer of JAX fecal material alone resulted in significantly slower tumor growth (Figure 2D), accompanied by increased tumor-specific T cell responses (Figure 2E) and infiltration of antigen-specific T cells into the tumor (Figure 2F), to the same degree as treatment with systemic αPD-L1 mAb. Combination treatment with both JAX fecal transfer and αPDL1 mAb improved tumor control (Figure 2D) and circulating tumor antigen-specific T cell responses (Figure 2E), while there was little additive effect on accumulation of activated T cells within the tumor microenvironment (Figure 2F). Consistent with these results, αPD-L1 therapy alone was significantly more efficacious in JAX mice compared to TAC mice (Figure 2G), which paralleled improved anti-tumor T cell responses (Figure 5C). These data indicate that the commensal microbial composition can influence spontaneous anti-tumor immunity as well as response to immunotherapy with αPD-L1 mAb.

To identify specific bacteria associated with protective anti-tumor immune responses, the fecal bacterial content in mice obtained from TAC mice, JAX mice, and JAX-fed and TAC-fed TAC mice we compared using the 16S ribosomal RNA (rRNA) miSeq Illumina platform.

Overall, 933.9 ± 55.2 taxa were identified in TAC mice and 653.4 ± 60 taxa were identified in JAX mice, demonstrating decreased species diversity in mice obtained from JAX. TAC mice that were orally administered JAX fecal material showed decreased taxa diversity (706.6 ± 117.9, p=0.006) similar to JAX mice, whereas TAC mice that were administered TAC fecal material did not show altered diversity (895.7 ± 118, p=1, Figure 3A). Principal coordinate analysis revealed that fecal samples analyzed from TAC mice that received JAX fecal material co-clustered separately from samples from control TAC mice and were more similar to samples obtained from JAX mice (Figure 3B, and became similar to samples obtained from sham and JAX feces-inoculated JAX mice (Figure 8A). In contrast, TAC-inoculated TAC mice did not change in community diversity relative to sham-inoculated TAC mice (p=0.4, ANOSIM). Analysis of similarity confirmed that TAC mice fed with JAX fecal material were more similar to each other than to TAC mice that were given TAC fecal material (p=0.008) or mice obtained from TAC (p=0.002). Reciprocal transfer of TAC fecal material into JAX hosts resulted in a statistically significant change in community diversity (p=0.003, ANOSIM), yet the distance of the microbial shift was smaller (Figure 8A).

Comparative analysis of specific bacterial taxa showed that 97 taxa were significantly more abundant in JAX mice relative to TAC mice (FDR<0.05) (Figure 8B), and 51 taxa were significantly increased in JAX-fed TAC mice relative to TAC-fed TAC mice (p<0.05). Only 32 taxa overlapped between these two comparisons, such that they were of greater abundance in both JAX mice and JAX-fed TAC mice. A significant association was observed for *Bifidobacterium*, which showed a positive association with anti-tumor T cell responses and increased in relative abundance over 400-fold in JAX-fed TAC mice (Figure 8C). Members belonging to several of these groups were similarly altered in JAX-fed TAC mice relative to sham- or TAC-inoculated TAC mice (Figure 8C). These included several unidentified taxa from the family S24-7 of the order *Bacteroidales*, one unassigned taxon, and four taxa with genus-level identifications, all of which are anaerobic gram-positive bacteria. Of these, the two most significant differentially abundant taxa belong to the *Bifidobacterium* genus, with the top *Bifidobacterium* taxon being over 200-fold more abundant in JAX relative to TAC (p=0.001), and similarly abundant in JAX-fed mice but not detected at all in TAC-fed TAC mice (p=0.01) (Figure 3C). Comparison of relative abundance of all taxa combined belonging to the *Bifidobacterium* genus yielded similar results (Figure 6A). Given that interactions between

bifidobacteria and the host immune system have been described previously (Lopez et al. International journal of food microbiology 138, 157-165 (2010).; Ménard et al. Applied and Environmental Microbiology 74, 660-666 (2008).; Dong et al. Early human development 86, 51-58 (2010).; herein incorporated by reference in their entireties), it was contemplated that members of this genus represent one source of the beneficial anti-tumor immune effects observed in JAX mice.

At the sequence level, *Bifidobacterium* operational taxonomic unit OTU_681370 showed the largest increase in relative abundance in JAX-fed TAC mice and the strongest association with anti-tumor T cell responses across all permutations (Figure 8D). This bacterium was further identified as most similar to *B. breve*, *B. longum* and *B. adolescentis* (99% identity). To test whether *Bifidobacterium* spp. may be sufficient to augment protective immunity against tumors, a commercially available cocktail of *Bifidobacterium* species was obtained, which included *B. breve* and *B. longum* and administered this by oral gavage, alone or in combination with αPD-L1, to TAC 7 recipients bearing established tumors. Analysis of fecal bacterial content revealed that the most significant change in response to *Bifidobacterium* inoculation occurred in the *Bifidobacterium* genus ($p=0.0009$, FDR=0.015, non-parametric t-test), with a 120-fold increase in OTU_681370 (Figure 9A), indicating that the commercial inoculum contained bacteria that were at least 97% identical to the taxon identified in JAX and JAX-fed TAC mice. An increase in *Bifidobacterium* could also be detected by quantitative PCR (Figure 9B).

Bifidobacterium-treated mice displayed significantly improved tumor control in comparison to non-*Bifidobacterium* treated counterparts (Figure 8E), which was accompanied by robust induction of tumor-specific T cells in the periphery (Figure 8F) and increased accumulation of antigen-specific CD8+ T cells within the tumor (Figure 8G and Figure 9C). These effects were durable for several weeks (Figure 9D-E).

The therapeutic effect of *Bifidobacterium* feeding was abrogated in CD8-depleted mice (Figure 10A), suggesting that the mechanism was not direct but rather through host anti-tumor T cell responses. Heat inactivation of the bacteria prior to oral administration also abrogated the therapeutic effect on tumor growth and reduced tumor-specific T cell responses to baseline (Figure 10B-D), suggesting that the anti-tumor effect requires live bacteria. As an alternative strategy, the therapeutic effect of *B. breve* and *B. longum* strains obtained from the ATCC was tested, which also showed significantly improved tumor control (Figure 11A). Administration of

Bifidobacterium to TAC mice inoculated with B16 parental tumor cells or MB49 bladder cancer cells also resulted in delayed tumor outgrowth (Figure 11, B and C respectively). Oral administration of *Lactobacillus murinus* to TAC mice, which was not among the overrepresented taxa in JAX-fed mice, had no effect on tumor growth (Figure 11D) or on tumor-specific T cell responses (Figure 11E), suggesting that modulation of anti-tumor immunity depends on the specific bacteria administered. Collectively, these data point to *Bifidobacterium* as a positive regulator of anti-tumor immunity in vivo.

Upon inoculation with *Bifidobacterium*, a small set of species were altered in parallel with *Bifidobacterium* (ANOSIM, p=0.003, Figure 12A), however, they largely did not resemble the changes observed with JAX-feces administration. Although reductions were observed (~2-10 fold) in members of the order *Clostridiales* as well as in butyrate-producing species upon *Bifidobacterium* inoculation, which could point to an inhibitory effect on the regulatory T cell compartment, no difference was observed in the frequency of $CD4^+$ $Foxp3^+$ T cells in tumors isolated from JAX and TAC mice (Figure 12B). Thus, it is unlikely that *Bifidobacterium* is acting primarily through modulation of the abundance of other bacteria.

It was next assessed whether translocation of *Bifidobacterium* was occurring into the mesenteric lymph nodes, spleen or tumor, however no *Bifidobacterium* was detected in any of the organs isolated from *Bifidobacterium*-gavaged tumor-bearing mice (Figure 12C). It was thus concluded that the observed systemic immunological effects are occurring independently of bacterial translocation.

To test whether *Bifidobacterium spp* may be sufficient to augment protective immunity against tumors, we administered a combination of four *Bifidobacterium* species was administered by oral gavage, alone or in combination with αPD-L1, to TAC recipients bearing 7-day established tumors. *Bifidobacterium*-treated mice displayed significantly improved tumor control in comparison to non-*Bifidobacterium* treated counterparts (Figure 3D), which was accompanied by robust induction of tumor-specific T cells in the periphery (Figure 3E) and markedly increased accumulation of antigen-specific $CD8^+$ T cells within the tumor (Figure 3F). This therapeutic effect was completely abrogated in CD8-depleted mice (Figure 3G), arguing that the mechanism was not direct but rather through host anti-tumor T cell responses. Heat inactivation of the bacteria prior to oral administration also abrogated the therapeutic effect on tumor growth and reduced tumor-specific T cell responses to baseline (Figure 6B-D), indicating

that the anti-tumor effect requires live bacteria. Administration of *Bifidobacterium* to TAC mice inoculated with B16 parental tumor cells or MB49 bladder cancer cells also resulted in delayed tumor outgrowth (Figure 6E-F). Oral administration of *Lactobacillus murinus* to TAC mice, which was not among the overrepresented taxa in JAX or JAX-fed mice, had no effect on tumor growth (Figure 6G) nor on tumor-specific T cell responses (Figure 6H), indicating that modulation of commensal bacterial communities through introduction of new bacteria in itself does not induce immunity to tumors, but rather immunity depends on the specific bacteria administered. Collectively, data identify *Bifidobacterium* as a positive regulator of anti-tumor immunity in vivo.

To interrogate mechanisms underlying the observed differences in T cell responses between JAX and TAC mice, we transferred CFSE-labeled SIY-specific 2C TCR Tg T cells into tumor-bearing mice and tested their proliferation and acquisition of IFN-γ production ex vivo (Figure 7A). $CD8^+$ SIY-specific 2C TCR Tg T cells exposed to tumors in JAX mice exhibited similar expansion in the tumor-draining lymph node as compared to their counterparts in TAC mice (Figure 7B). However, they produced markedly greater IFN-γ in both the tumor draining lymph node and the spleen of JAX tumor-bearing mice (Figure 4A and 4B), suggesting that signals upstream of T cells in the JAX environment enhanced acquisition of T cell effector function. These data indicated an improvement in immune responses upstream of T cells, at the level of host dendritic cells (DCs). Genome-wide transcriptional profiling of early tumor-infiltrating DCs isolated from JAX, TAC and *Bifidobacterium*-treated TAC mice was employed (Figure 13A). In total, there were 761 gene transcripts upregulated by ≥1.5-fold in both JAX and *Bifidobacterium*-treated TAC-derived DCs relative to DCs from untreated TAC mice (Figure 4C). Pathway analysis identified cytokine-cytokine receptor interaction, T cell activation, and positive regulation of mononuclear cell proliferation as significantly enriched pathways among upregulated genes (Figure 4C and Figure 13B). Many of these genes have been shown to be critical for anti-tumor responses including those involved in $CD8^+$ T cell activation and costimulation (*H2-m2*(MHC-I), *Cd40*, *Cd70*, *Icam1)*) (Mackey et al. Journal of immunology (Baltimore, Md. : 1950) 161, 2094-2098 (1998).; Scholer et al. Immunity 28, 258-270 (2008).; Bak et al. Journal of immunology (Baltimore, Md. : 1950) 189, 1708-1716 (2012).; herein incorporated by reference in their entireties), DC maturation (*Relb, Ifngr2*) (Pan et al. Immunology letters 94, 141-151 (2004).; Pettit et al. Journal of immunology (Baltimore, Md. :

1950) 159, 3681-3691 (1997).; herein incorporated by reference in their entireties), antigen processing and cross presentation (*Tapbp*, *Rab27a*, *Slc11a1*) (Compeer et al. Frontiers in Immunology 3, (2012).; Jancic et al. Nature cell biology 9, 367-378 (2007).; Stober et al. Infection and Immunity 75, 5059-5067 (2007).; herein incorporated by reference in their entireties), chemokine–mediated recruitment of immune cells to the tumor microenvironment (*Cxcl9*, *Cx3cl1*, *Cxcr4*) (Kabashima et al. The American Journal of Pathology 171, 1249-1257 (2007).; Nukiwa et al. European journal of immunology 36, 1019-1027 (2006).; Zhang et al. New England Journal of Medicine 348, 203-213 (2003).; herein incorporated by reference in their entireties) and type I interferon signaling (*Irf1*, *Ifnar2*, *Oas2*, *Ifi35*, *Ifitm1*) (Fuertes et al. The Journal of experimental medicine 208, 2005-2016 (2011).; Woo et al. Immunity 41, 830-842 10.; herein incorporated by reference in their entireties) (Figure 4D). Expression of these genes was also strongly induced in murine bone marrow-derived DCs stimulated with *Bifidobacterium in vitro*. Taken together, these data indicate that commensal bacteria-derived (e.g., *Bifidobacterium*-derived) signals modulate the activation of innate antigen-presenting cells, which in turn support improved activation of tumor antigen-specific $CD8^+$ T cells.

To test whether functional differences in DCs isolated from TAC, JAX and *Bifidobacterium*-treated TAC mice could be sufficient to explain the differences in T cell priming observed in vivo, DCs were purified from lymphoid tissues of naïve TAC, JAX, and *Bifidobacterium*-treated TAC mice and tested their ability to induce CFSE-labeled $CD8^+$ SIY^- specific 2C TCR Tg T cell proliferation and acquisition of IFN-γ production *in vitro*. DCs purified from JAX and *Bifidobacterium*-treated TAC mice induced 2C T cell proliferation at lower antigen concentration compared to DCs purified from naïve TAC mice (Figure 14, A and B). Furthermore, at all antigen concentrations, JAX-derived DCs elicited elevated levels of T cell IFN-γ production (Figure 4E and Figure 14A). Similar effects were observed upon oral administration of *Bifidobacterium* to TAC mice prior to DC isolation (Figure 4E and Figure 14A). Taken together, these data indicate that commensal *Bifidobacterium*-derived signals modulate the activation of DCs in the steady state, which in turn supports improved effector function of tumor-specific $CD8^+$ T cells.

Experiments conducted during development of embodiments herein demonstrate an unexpected role for commensal microflora (e.g., *Bifidobacterium*) in enhancing anti-tumor immunity. These data support the idea that one source of inter-subject heterogeneity with regard

to spontaneous anti-tumor immunity and therapeutic effects of antibodies targeting the PD-1/PD-L1 axis may be the specific composition of gut microbes, which can be manipulated for therapeutic benefit.

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CLAIMS

We claim:

1. A method of treating cancer in a human subject comprising administering to the subject an immune checkpoint inhibitor and a bacterial formulation comprising bacteria of the genera *Bifidobacterium*.

2. The method of claim 1, wherein at least 50% of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*.

3. The method of claim 1, wherein at least 90% of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*.

4. The method of claim 1, wherein the bacteria of the genus *Bifidobacterium* comprise bacteria of the species *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium asteroides, Bifidobacterium boum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium cuniculi, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium indicum, Bifidobacterium inopinatum, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium pseudolongum, Bifidobacterium pullorum, Bifidobacterium psychraerophilum, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium scardovii, Bifidobacterium simiae, Bifidobacterium subtile, Bifidobacterium therammcidophilum, Bifidobacterium thermophilum, Bifidobacterium tsurumiense, Bifidobacterium urinalis* or *Bifidobacterium sp.*.

5. The method of claim 1, wherein the bacterial formulation is administered by oral administration or rectal administration.

6. The method of claim 5, wherein the bacterial formulation is administered by oral administration.

7. The method of claim 1, wherein the bacterial formulation comprises at least $5x10^6$ CFU of bacteria of the genera *Bifidobacterium*.

8. The method of claim 1, wherein the bacterial formulation is administered to the subject in two or more doses.

9. The method of claim 9, wherein the administration of the two or more doses are separated by at least 1 week.

10. The method of claim 1, further comprising administering to the subject an antibiotic prior to the administration of the bacterial formulation.

11. The method of claim 10, wherein the antibiotic is administered to the subject at least 1 day before the bacterial formulation is administered to the subject.

12. The method of claim 1, wherein the immune checkpoint inhibitor is a protein or polypeptide that binds to an immune checkpoint protein.

13. The method of claim 12, wherein the immune checkpoint protein is CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA.

14. The method of claim 13, wherein the immune checkpoint protein is PD-1 or PD-L1.

15. The method of claim 1, wherein the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to an immune checkpoint protein.

16. The method of claim 15, wherein the immune checkpoint protein is CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA.

17. The method of claim 16, wherein the immune checkpoint protein is PD-1 or PD-L1.

18. The method of claim 1, wherein the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT Ol l, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010.

19. The method of claim 1, wherein the immune checkpoint inhibitor is administered by intravenous injection, intramuscular injection, intratumoral injection or subcutaneous injection.

20. A method of treating cancer in a human subject comprising administering to the subject a bacterial formulation comprising at least $5x10^6$ CFU of bacteria of the genera *Bifidobacterium*, wherein at least 50% of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*.

21. The method of claim 20, wherein at least 90% of the bacteria in the bacterial formulation are of the genera *Bifidobacterium*.

22. The method of claim 20, wherein the bacteria of the genus *Bifidobacterium* comprise bacteria of the species *Bifidobacterium lactis, Bifidobacterium bifidium, Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium asteroides, Bifidobacterium boum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium cuniculi, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium indicum, Bifidobacterium inopinatum, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium pseudolongum, Bifidobacterium pullorum, Bifidobacterium psychraerophilum, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium scardovii, Bifidobacterium simiae, Bifidobacterium subtile, Bifidobacterium therammcidophilum, Bifidobacterium thermophilum, Bifidobacterium tsurumiense, Bifidobacterium urinalis* or *Bifidobacterium sp.*.

23. The method of claim 20, wherein the bacterial formulation is administered by oral administration or rectal administration.

24. The method of claim 23, wherein the bacterial formulation is administered by oral administration.

25. The method of claim 20, wherein the bacterial formulation is administered to the subject in two or more doses.

26. The method of claim 20, further comprising administering to the subject an antibiotic before the bacterial formulation is administered to the subject.

27. The method of claim 20, further comprising administering to the subject an immune checkpoint inhibitor.

28. The method of claim 27, wherein the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA.

29. The method of claim 27, wherein the immune checkpoint inhibitor is an antibody or antigen binding fragment thereof that binds to PD-1 or PD-L1.

30. The method of claim 27, wherein the immune checkpoint inhibitor is nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, BMS-936558, MK-3475, CT Ol l, MPDL3280A, MEDI-4736, MSB-0020718C, AUR-012 and STI-A1010.

ABSTRACT

Provided herein are methods of treatment and/or prevention of cancer by manipulation of commensal microflora. In particular, the amount, identity, presence, and/or ratio of microflora (e.g., gut microflora) in a subject is manipulated to facilitate one or more co-treatments.

FIG. 1A

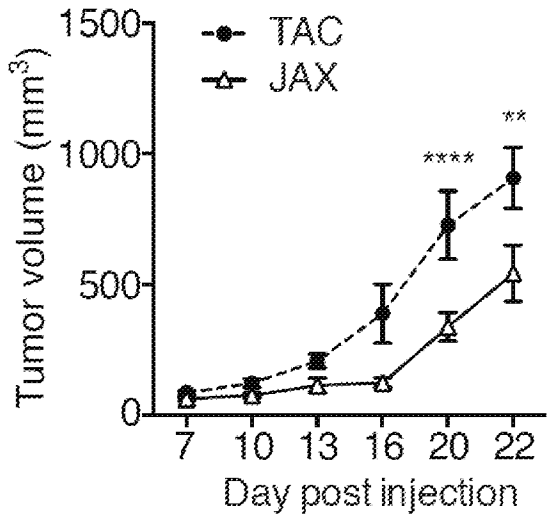


FIG. 1B

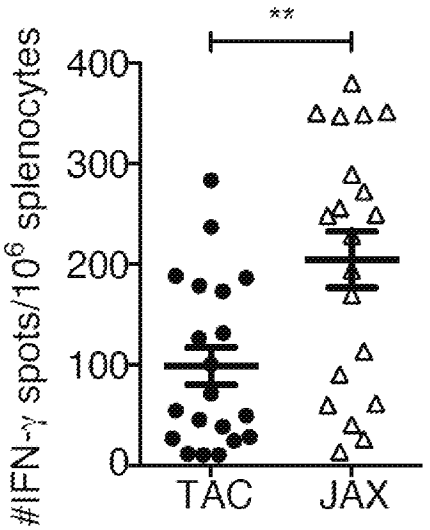


FIG. 1C

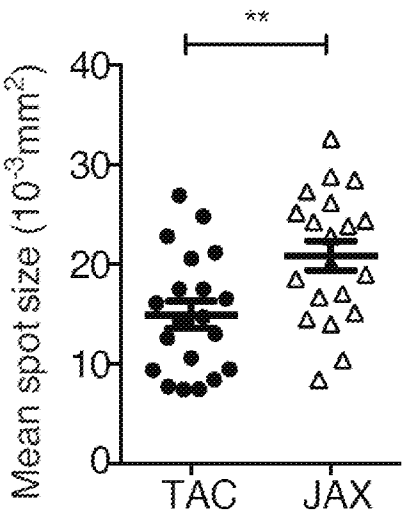
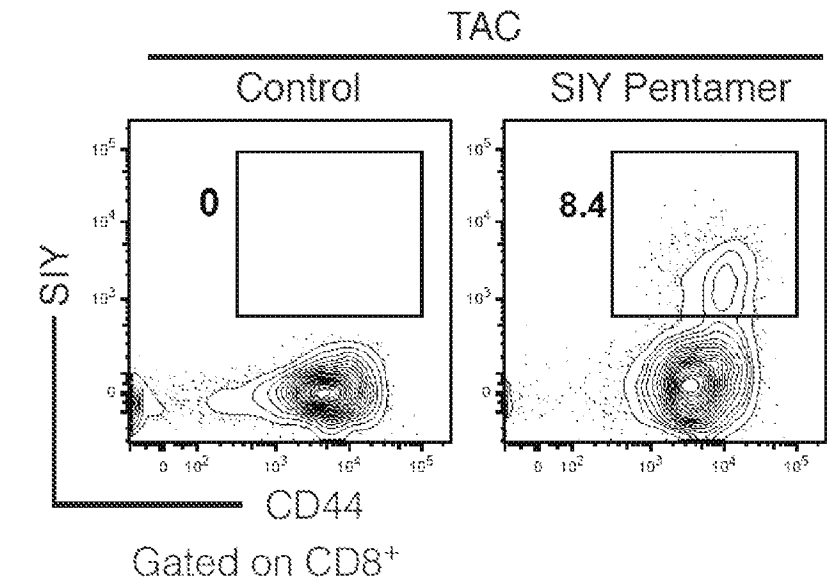
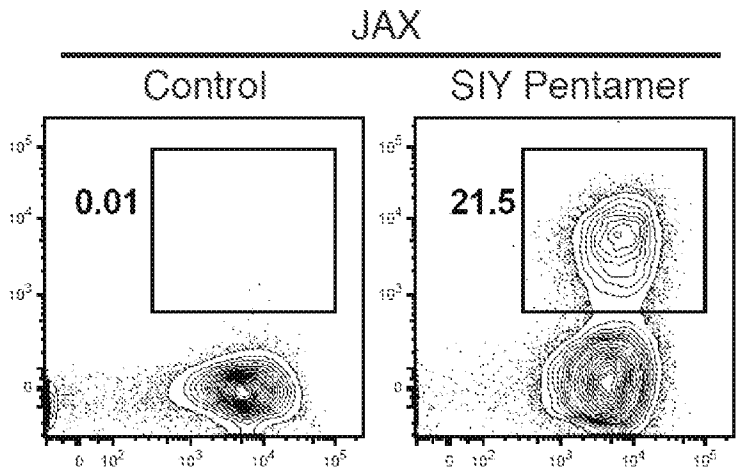
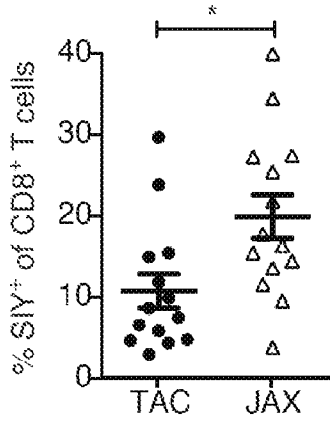


FIG. 1D


TAC
Control
SIY Pentamer
0
8.4
SIY
CD44
Gated on CD8+


JAX
Control
SIY Pentamer
0.01
21.5


*
% SIY+ of CD8+ T cells
40
30
20
10
0
TAC
JAX

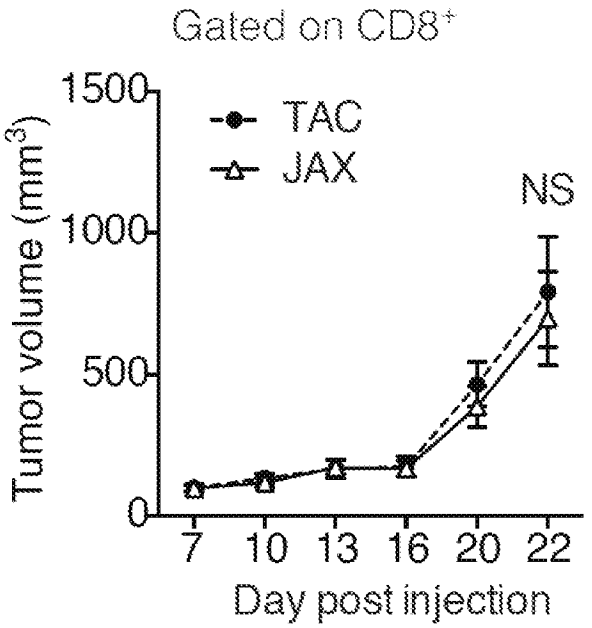


FIG. 1E

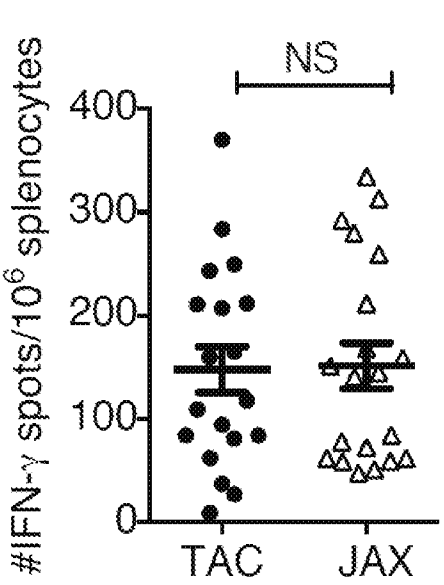


FIG. 1F

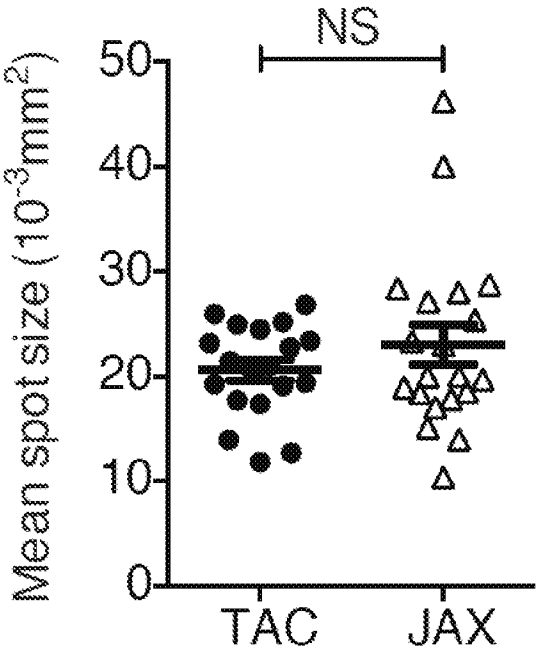


FIG. 1G

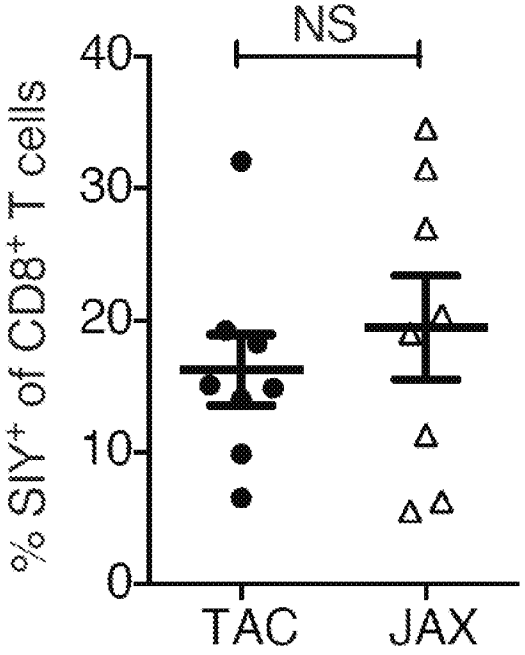


FIG. 1H

FIG. 2A

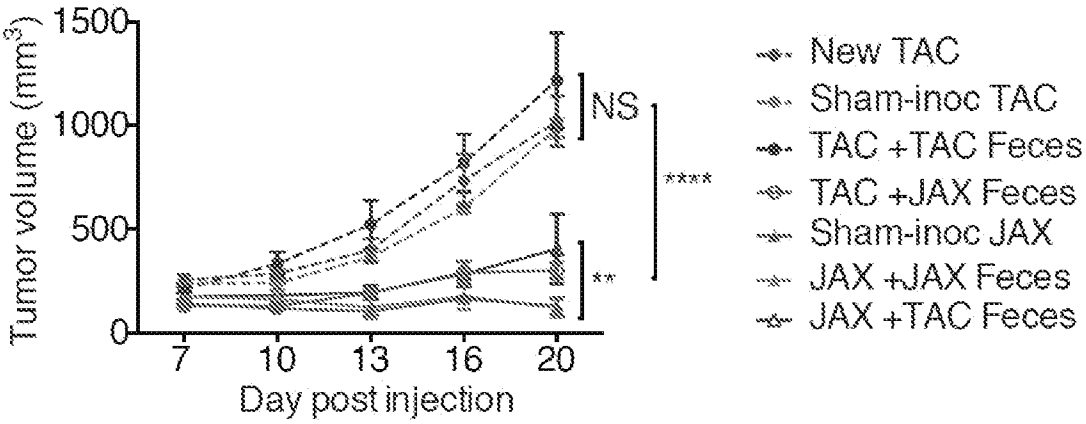

Tumor volume (mm3)
1500
1000
500
0
7
10
13
16
20
Day post injection
NS
****
**
New TAC
Sham-inoc TAC
TAC +TAC Feces
TAC +JAX Feces
Sham-inoc JAX
JAX +JAX Feces
JAX +TAC Feces

FIG. 2B

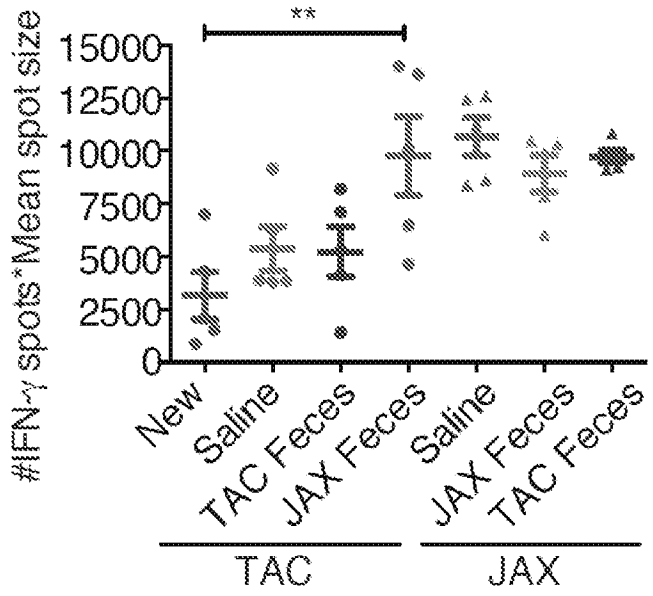
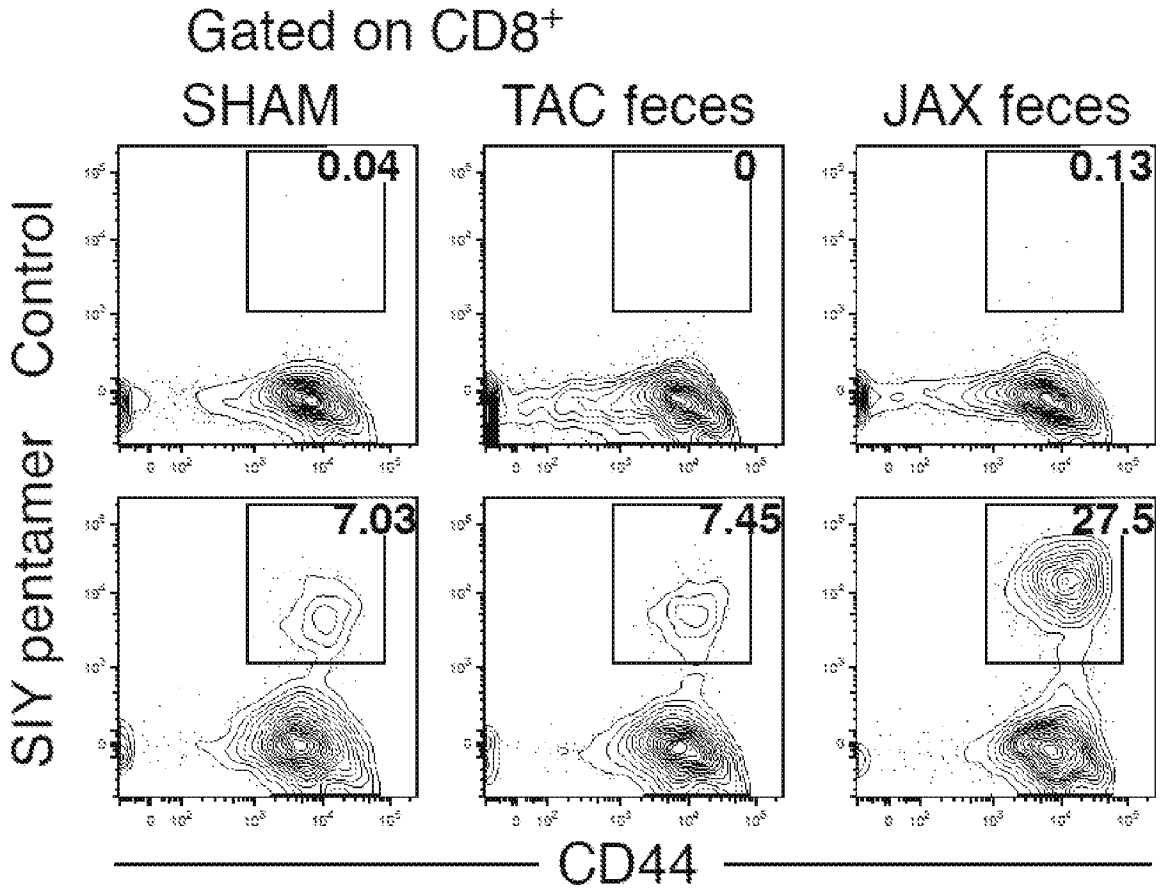

#IFN-γ spots*Mean spot size
15000
12500
10000
7500
5000
2500
0
**
New
Saline
TAC Feces
JAX Feces
Saline
JAX Feces
TAC Feces
TAC
JAX

FIG. 2C


Gated on CD8+
SHAM
TAC feces
JAX feces
SIY pentamer Control
0.04
0
0.13
7.03
7.45
27.5
CD44

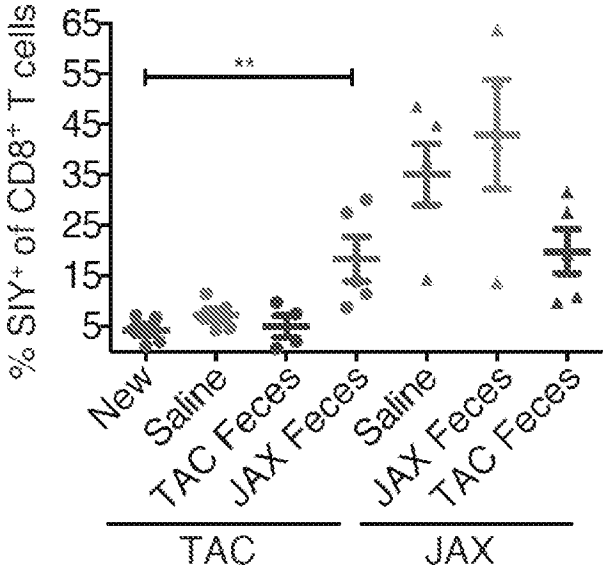

% SIY+ of CD8+ T cells
65
55
45
35
25
15
5
**
New
Saline
TAC Feces
JAX Feces
Saline
JAX Feces
TAC Feces
TAC
JAX

FIG. 2D

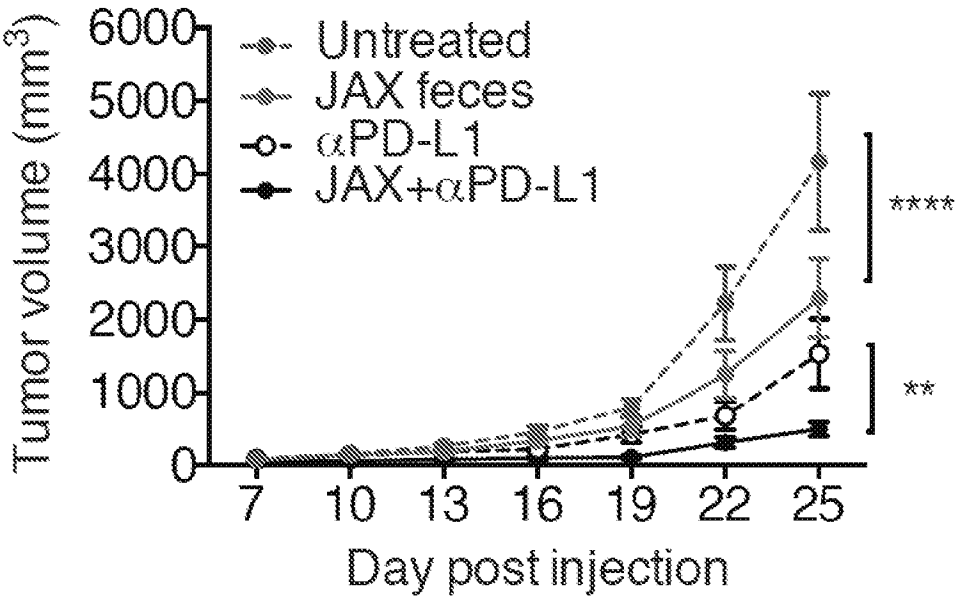

Tumor volume (mm3)
6000
5000
4000
3000
2000
1000
0
Untreated
JAX feces
αPD-L1
JAX+αPD-L1
****
**
7
10
13
16
19
22
25
Day post injection

FIG. 2E

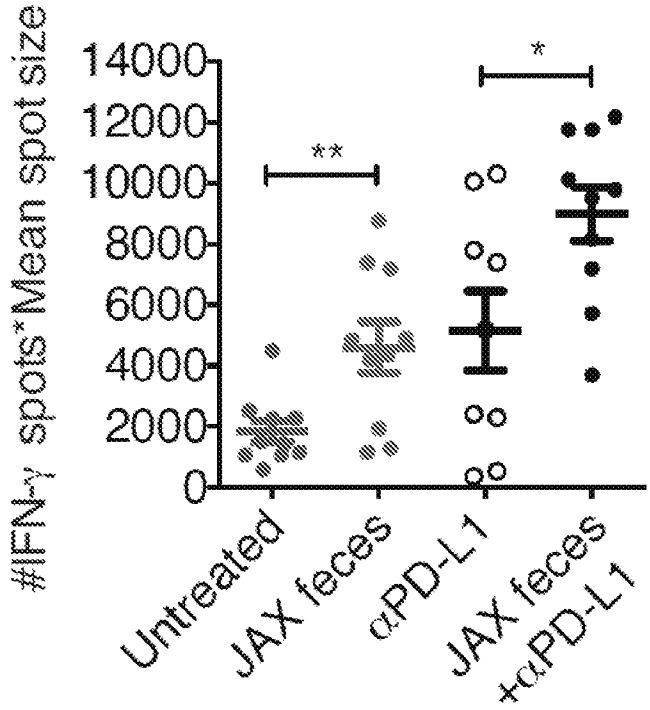

#IFN-γ spots*Mean spot size
14000
12000
10000
8000
6000
4000
2000
0
**
*
Untreated
JAX feces
αPD-L1
JAX feces
+αPD-L1

FIG. 2F

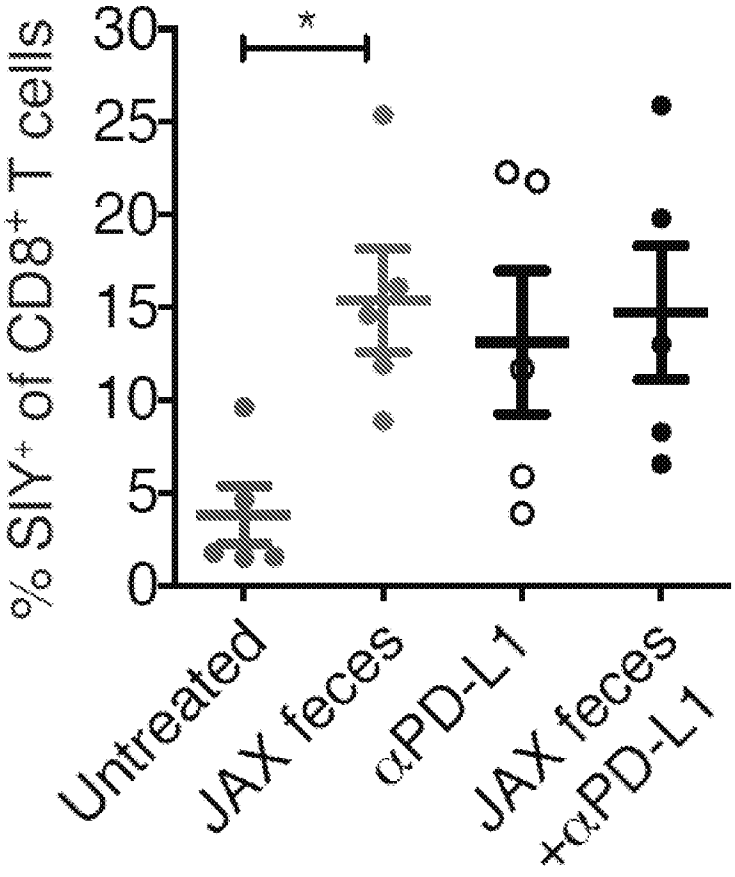

% SIY+ of CD8+ T cells
30
25
20
15
10
5
0
*
Untreated
JAX feces
αPD-L1
JAX feces
+αPD-L1

FIG. 2G

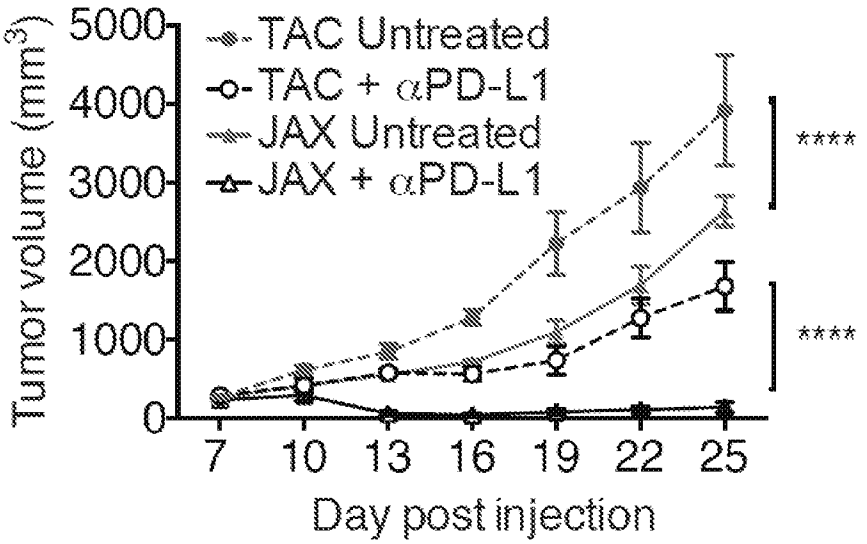

Tumor volume (mm3)
5000
4000
3000
2000
1000
0
TAC Untreated
TAC + αPD-L1
JAX Untreated
JAX + αPD-L1
****
****
7
10
13
16
19
22
25
Day post injection

FIG. 3A

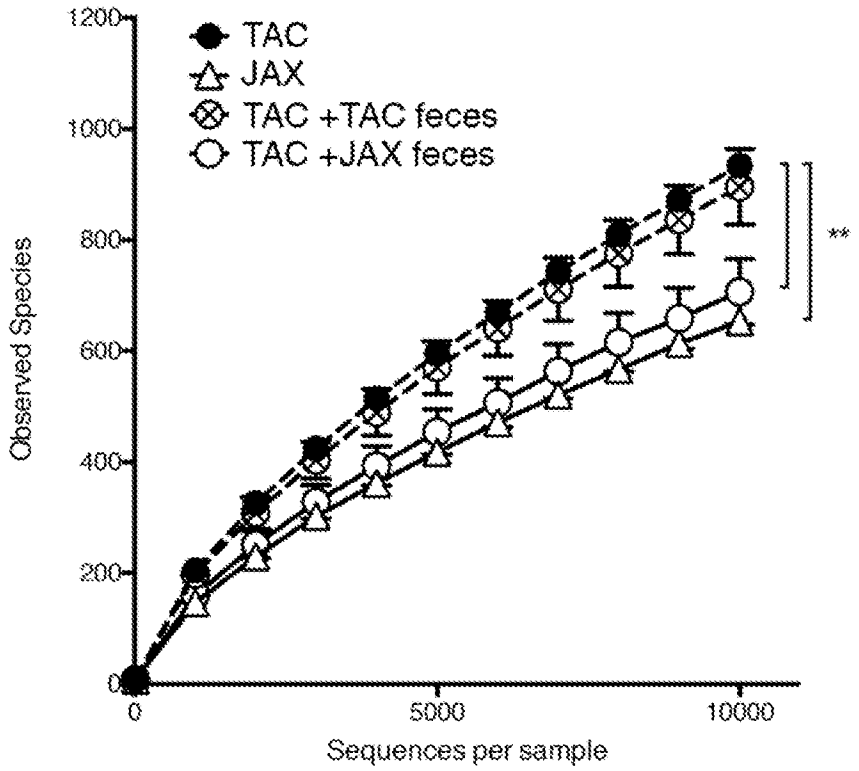

TAC
JAX
TAC +TAC feces
TAC +JAX feces
Observed Species
1200
1000
800
600
400
200
0
0
5000
10000
Sequences per sample
**

FIG. 3B

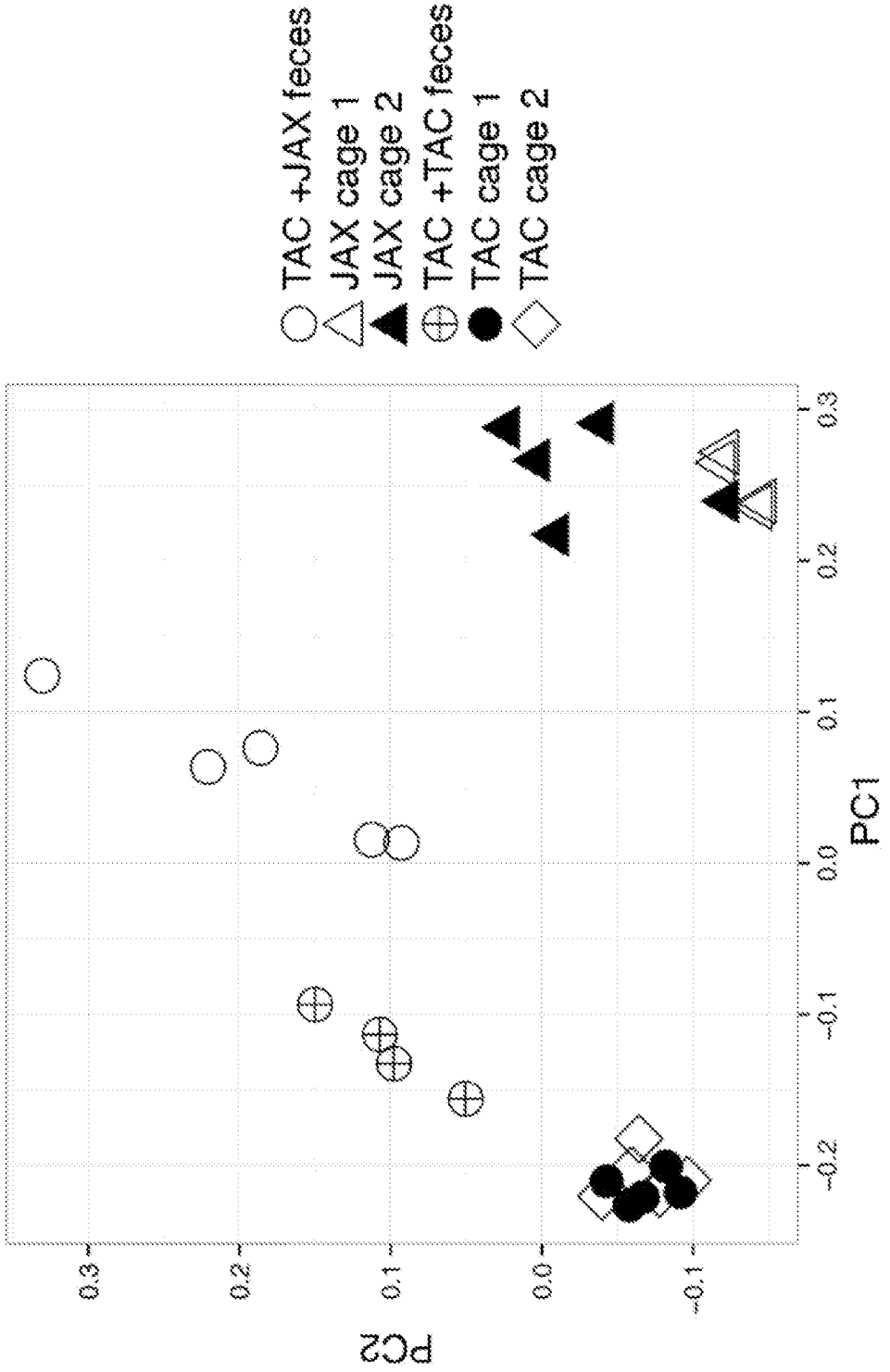

TAC +JAX feces
JAX cage 1
JAX cage 2
TAC +TAC feces
TAC cage 1
TAC cage 2
PC2
PC1
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.1
0.0
0.1
0.2
0.3

FIG. 3C

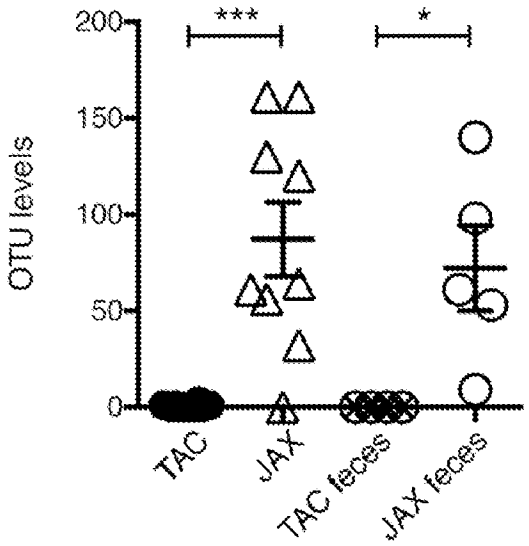


FIG. 3D

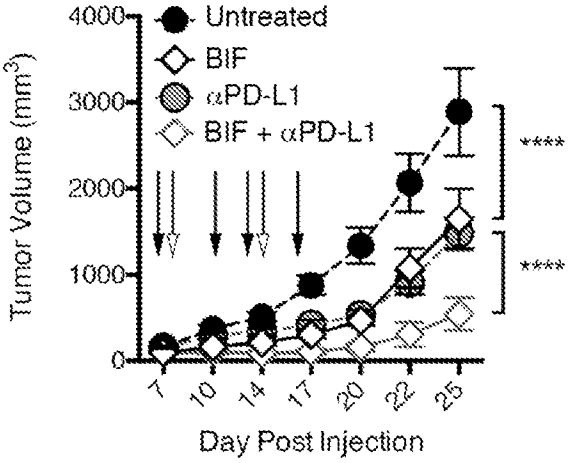


FIG. 3E

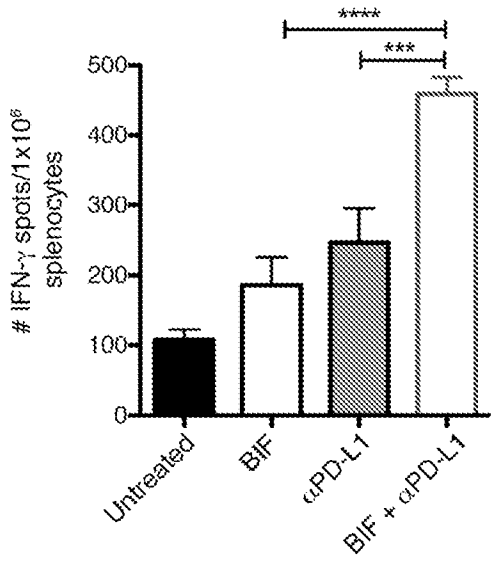


FIG. 3F

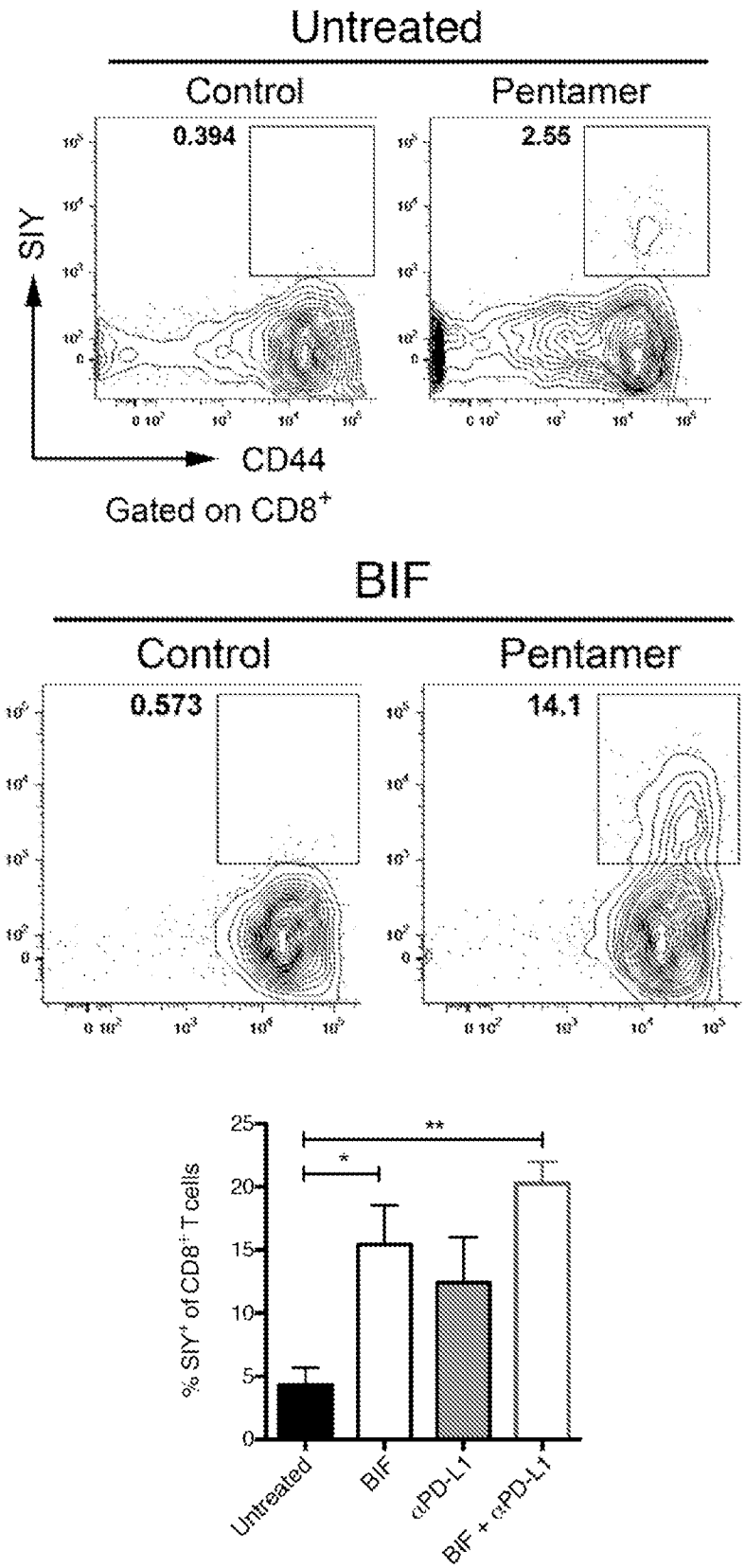

Untreated
Control
Pentamer
0.394
2.55
SIY
CD44
Gated on CD8+
BIF
Control
Pentamer
0.573
14.1
% SIY+ of CD8+ T cells
25
20
15
10
5
0
*
**
Untreated
BIF
αPD-L1
BIF + αPD-L1

FIG. 3G

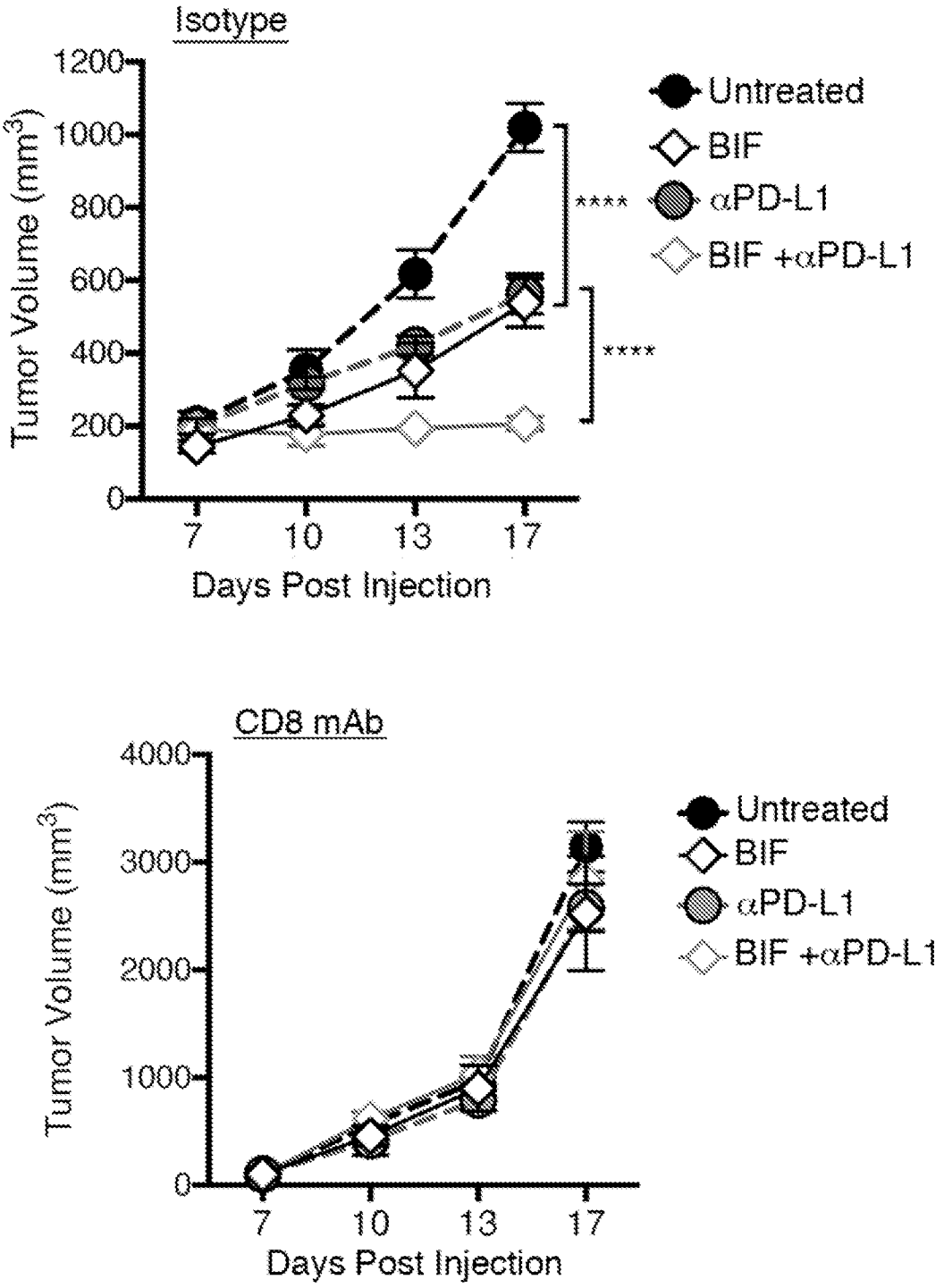

Isotype
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
7
10
13
17
Days Post Injection
Untreated
BIF
αPD-L1
BIF +αPD-L1
****
****
CD8 mAb
Tumor Volume (mm3)
4000
3000
2000
1000
0
7
10
13
17
Days Post Injection
Untreated
BIF
αPD-L1
BIF +αPD-L1

FIG. 4A

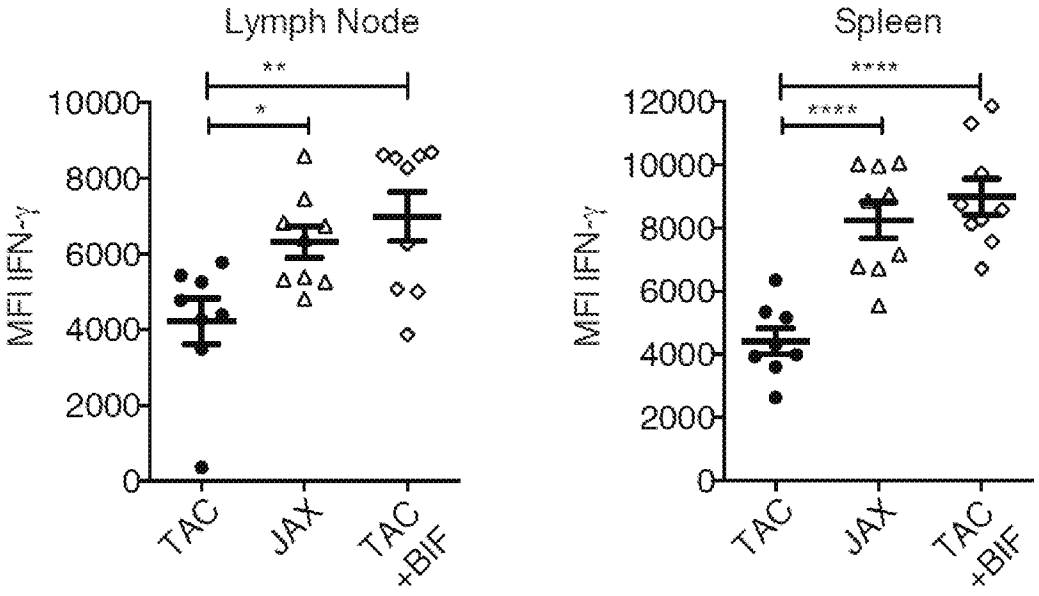

Lymph Node
Spleen
MFI IFN-γ
10000
8000
6000
4000
2000
0
12000
**
*
****
TAC
JAX
TAC +BIF

FIG. 4B

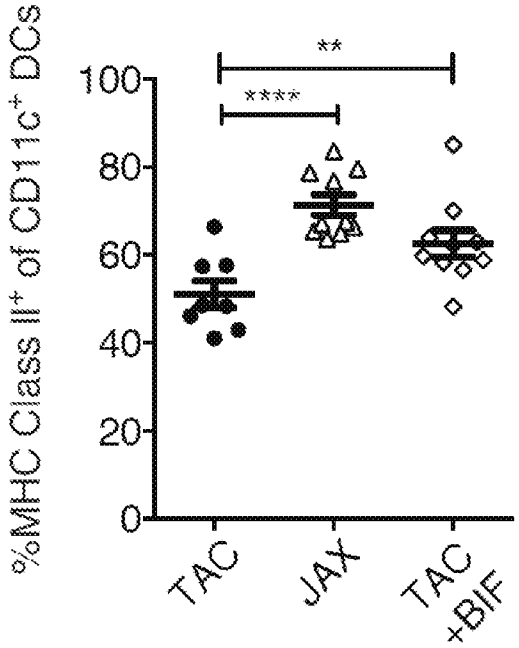

%MHC Class II+ of CD11c+ DCs
100
80
60
40
20
0
**
****
TAC
JAX
TAC +BIF

FIG. 4C

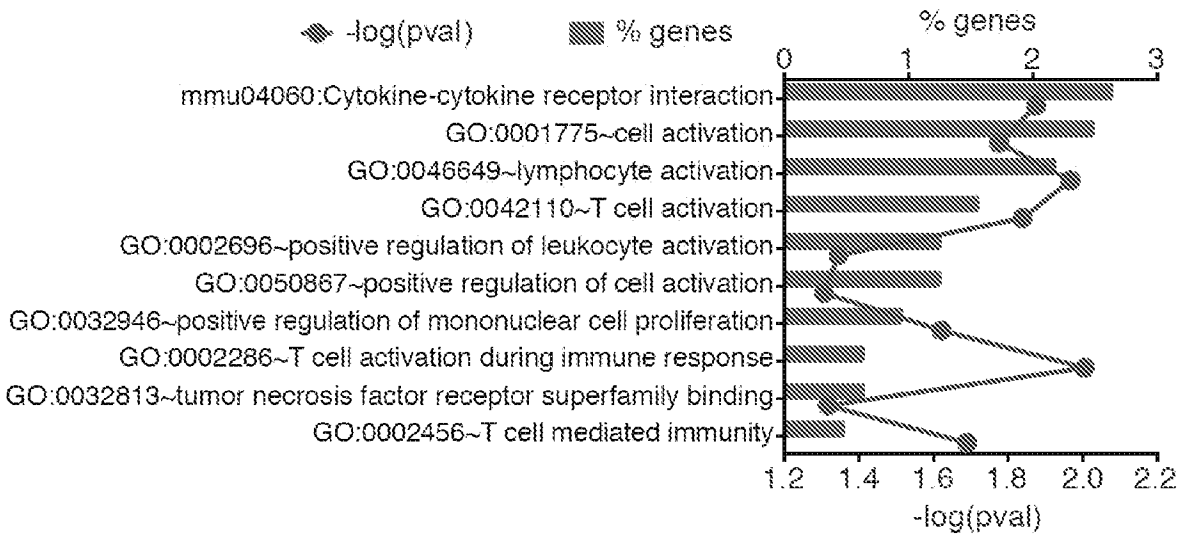

-log(pval)
% genes
% genes
0
1
2
3
mmu04060:Cytokine-cytokine receptor interaction
GO:0001775~cell activation
GO:0046649~lymphocyte activation
GO:0042110~T cell activation
GO:0002696~positive regulation of leukocyte activation
GO:0050867~positive regulation of cell activation
GO:0032946~positive regulation of mononuclear cell proliferation
GO:0002286~T cell activation during immune response
GO:0032813~tumor necrosis factor receptor superfamily binding
GO:0002456~T cell mediated immunity
1.2
1.4
1.6
1.8
2.0
2.2
-log(pval)

FIG. 4E

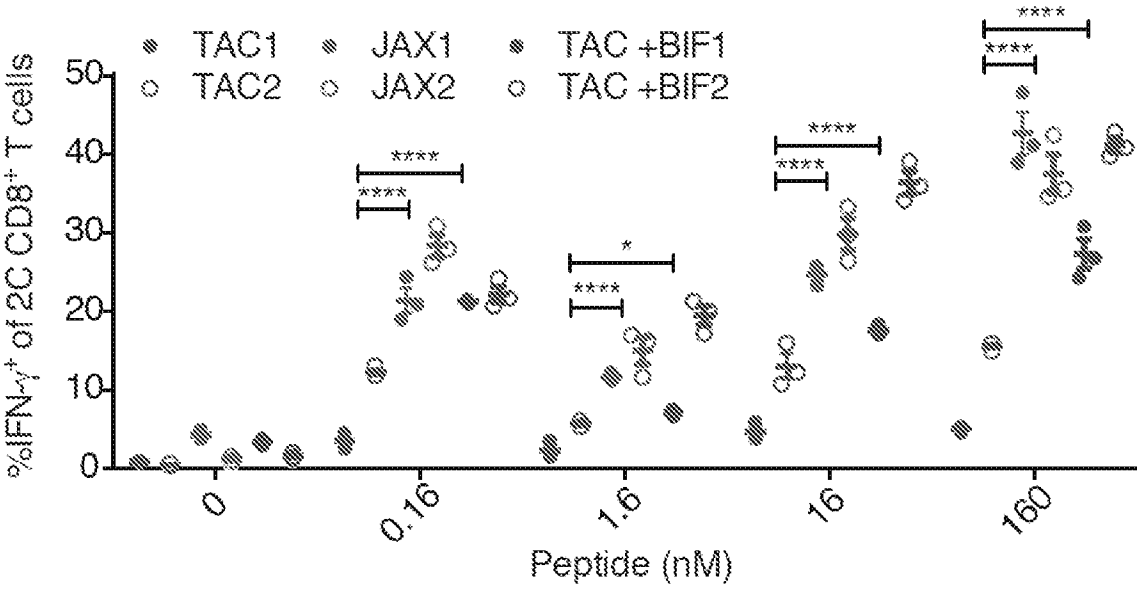
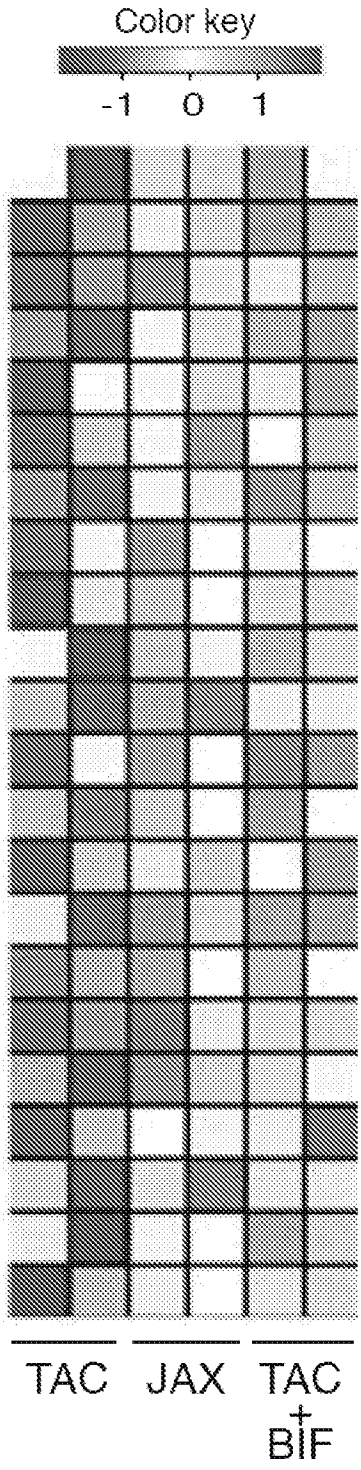

TAC1
TAC2
JAX1
JAX2
TAC +BIF1
TAC +BIF2
%IFN-γ+ of 2C CD8+ T cells
50
40
30
20
10
0
****
****
*
****
****
****
****
0
0.16
1.6
16
160
Peptide (nM)

FIG. 4D



Gene	JAX/TAC	BIF/TAC
H2m2	2.18	6.99
Cd40	1.58	2.11
Cd70	9.67	4.33
Icam1	1.57	2.00
Tnfrsf18	1.51	1.68
Tnfsf14	2.05	1.70
Relb	1.79	2.54
Ifngr2	3.10	1.71
Tapbp	42.40	14.97
Rab27a	2.55	2.96
Slc11a1	3.46	1.79
Cxcl9	2.64	1.53
Cx3cl1	3.96	6.36
Cxcr4	2.20	2.41
Irf1	1.55	2.44
Ifnar2	2.70	2.33
Oas2	1.68	1.51
Ifi35	1.92	1.57
Ifitm1	2.82	5.00
Azi2	7.71	3.04
Ikbke	1.60	2.03
Ulk1	5.78	3.51

FIG. 5A

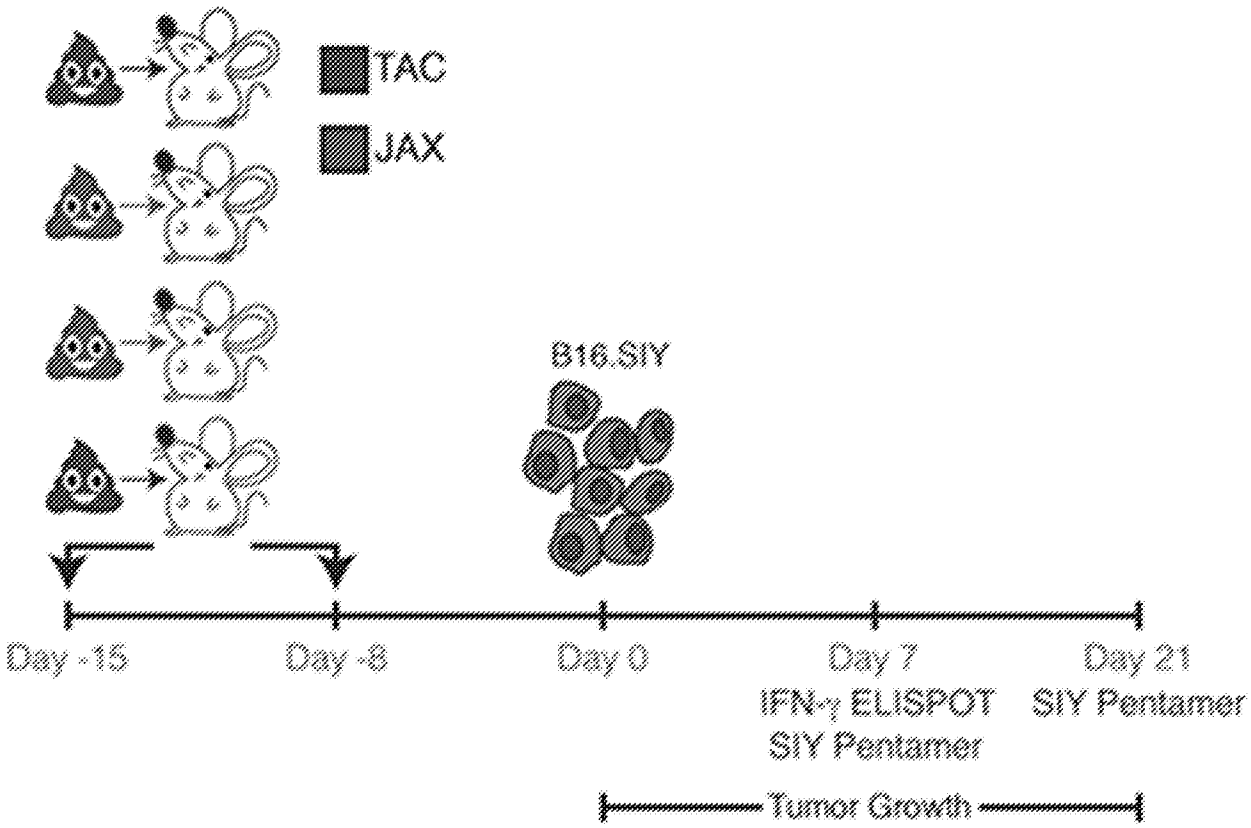

TAC
JAX
B16.SIY
Day -15
Day -8
Day 0
Day 7
Day 21
IFN-γ ELISPOT
SIY Pentamer
SIY Pentamer
Tumor Growth

FIG. 5B

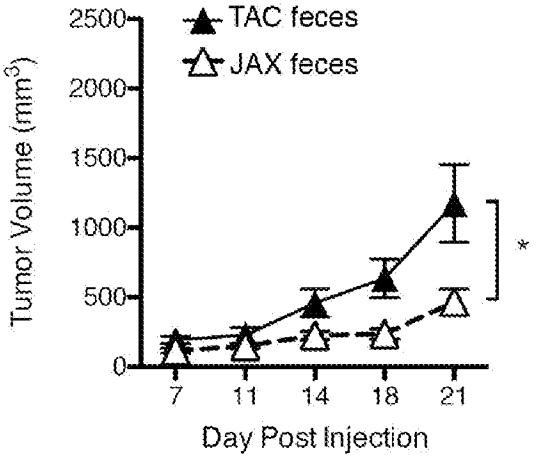


FIG. 5C

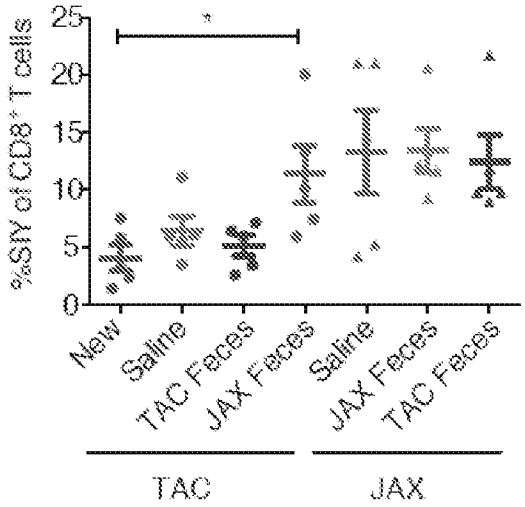


FIG. 5D

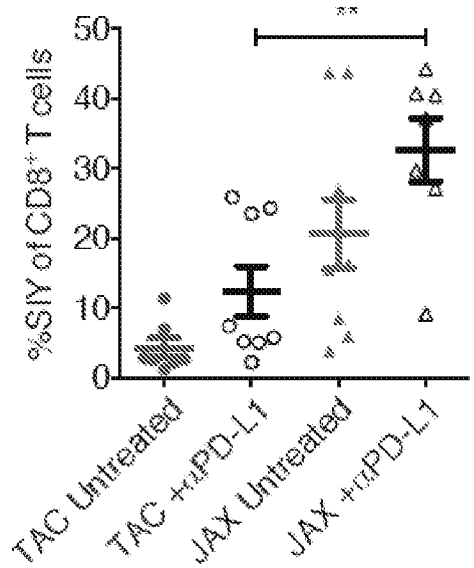


FIG. 6A

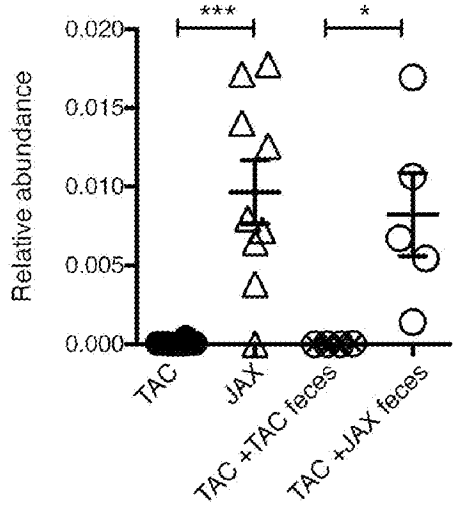


FIG. 6B

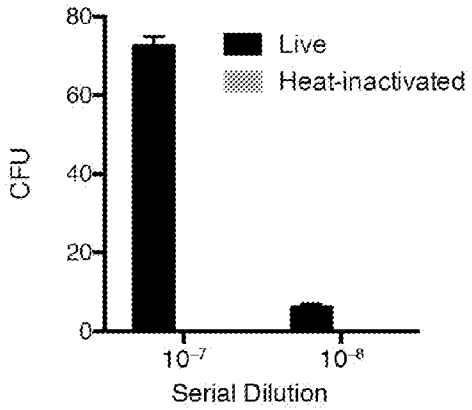


FIG. 6C

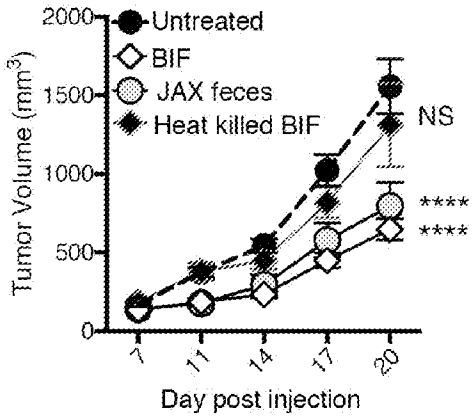


FIG. 6D

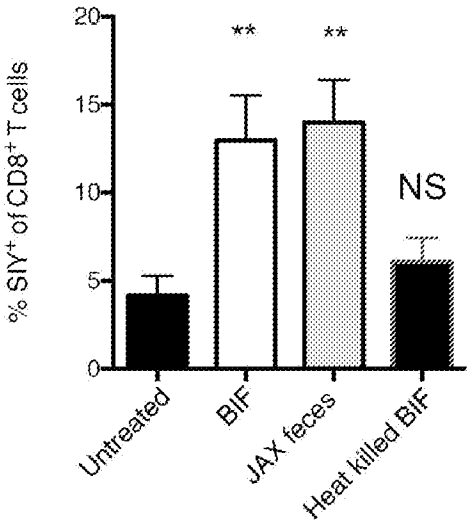


FIG. 6E

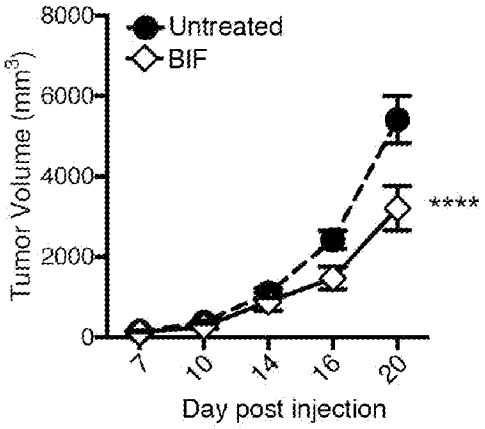


FIG. 6F

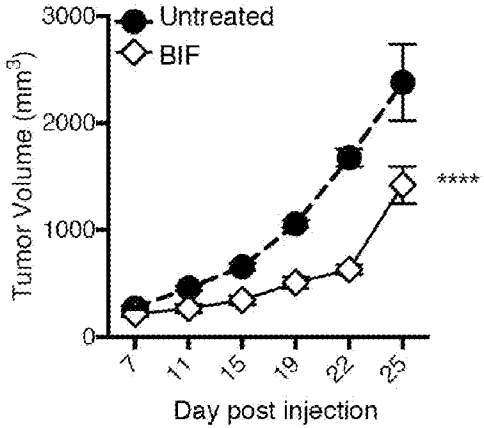


FIG. 6G

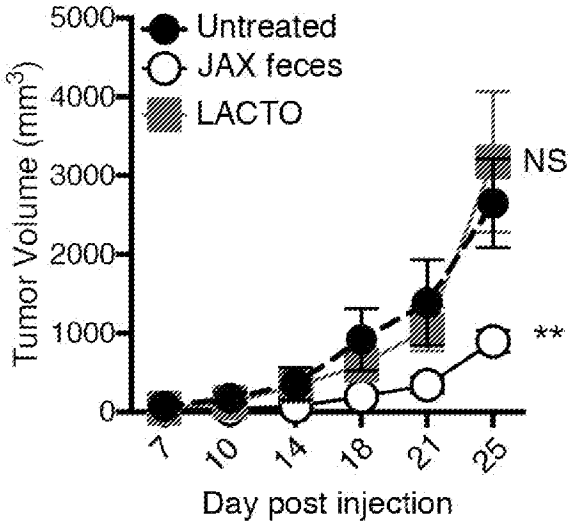

Untreated
JAX feces
LACTO
Tumor Volume (mm3)
5000
4000
3000
2000
1000
0
NS
**
7
10
14
18
21
25
Day post injection

FIG. 6H

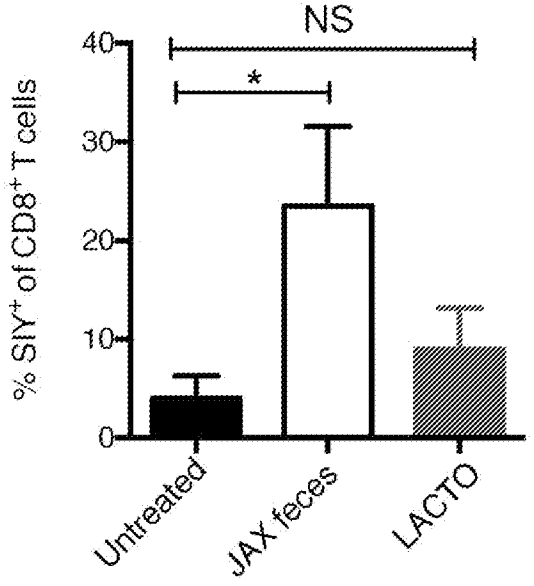
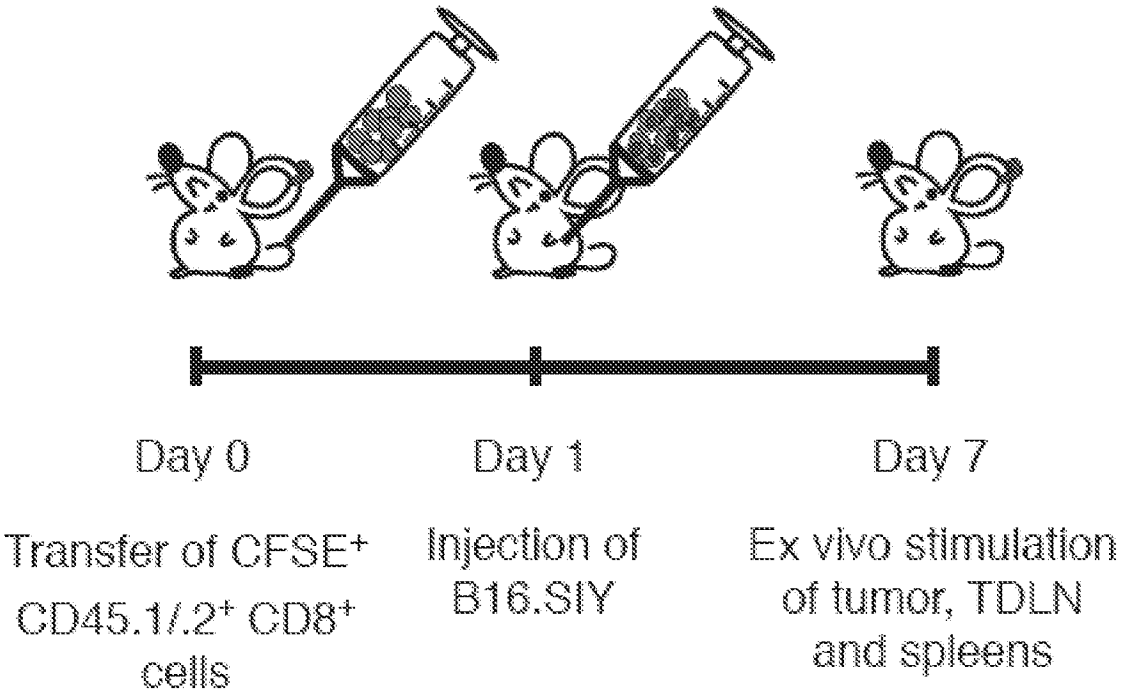
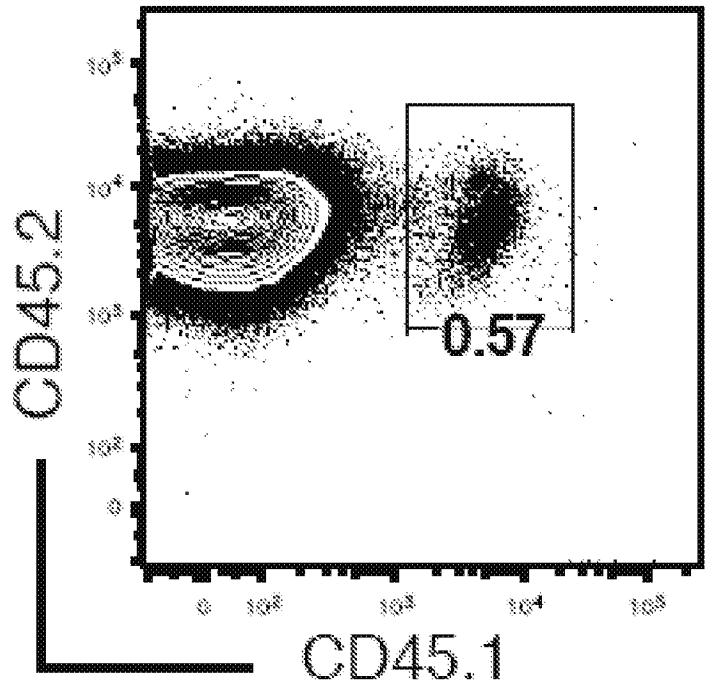

NS
*
% SIY+ of CD8+ T cells
40
30
20
10
0
Untreated
JAX feces
LACTO

FIG. 7A


Day 0
Day 1
Day 7
Transfer of CFSE+ CD45.1/.2+ CD8+ cells
Injection of B16.SIY
Ex vivo stimulation of tumor, TDLN and spleens


CD45.2
CD45.1
0.57

IFN-γ
CFSE
TAC
19.1
JAX
5.1
TAC + BIF
3.93
% of 2C CD8+ T cells with undiluted CFSE
0
20
40
60
TAC
JAX
BIF
Lymph Node

FIG. 7B

FIG. 8A

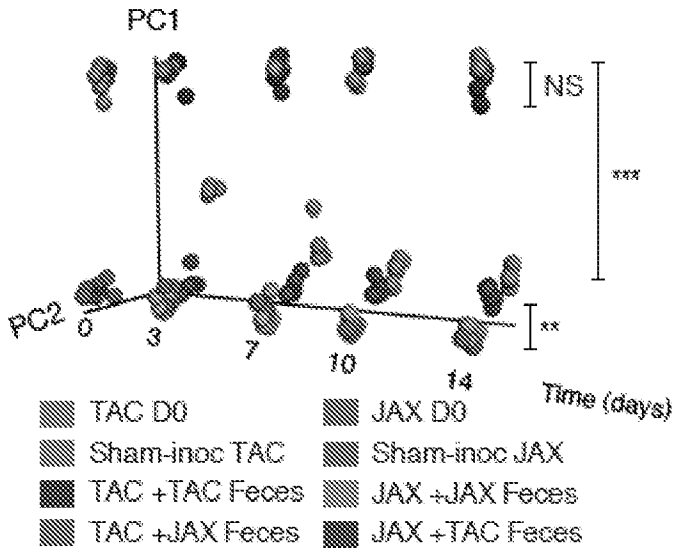

PC1
PC2
NS
***
**
0
3
7
10
14
Time (days)
TAC D0
Sham-inoc TAC
TAC +TAC Feces
TAC +JAX Feces
JAX D0
Sham-inoc JAX
JAX +JAX Feces
JAX +TAC Feces

FIG. 8B

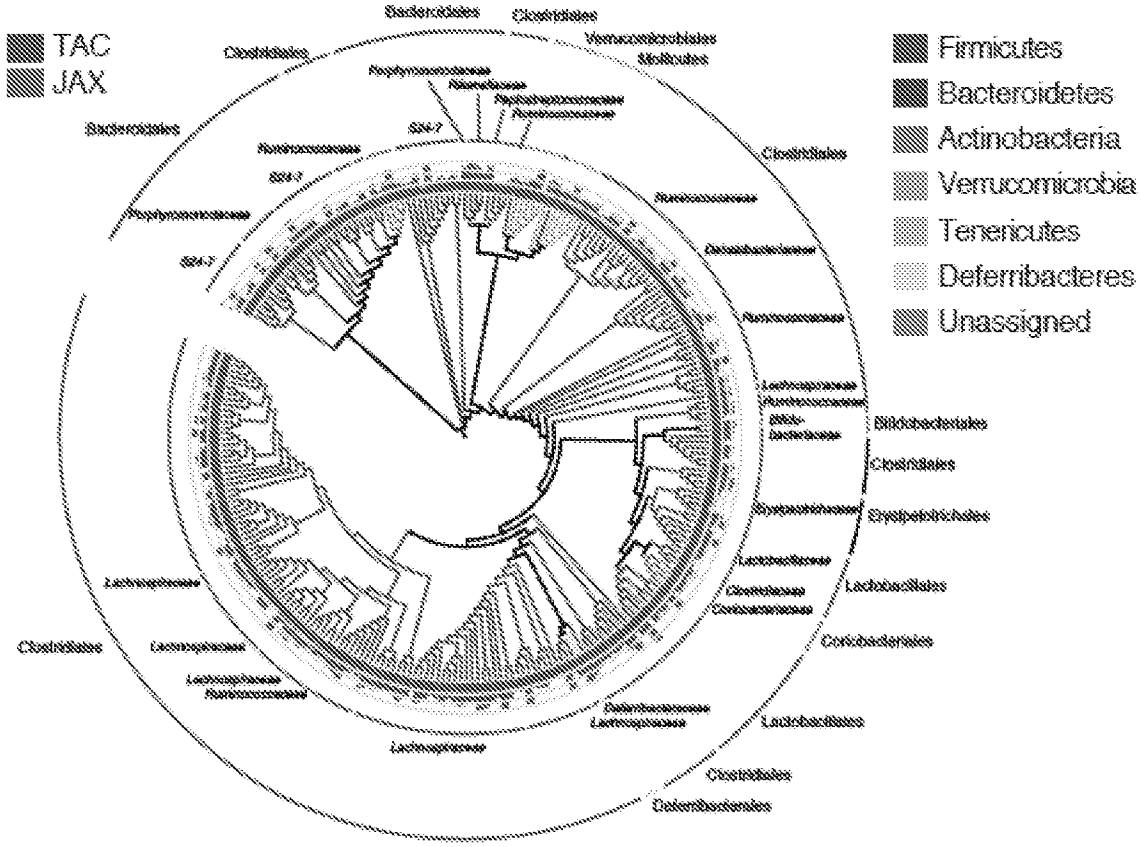

TAC
JAX
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Tenericutes
Deferribacteres
Unassigned

FIG. 8C

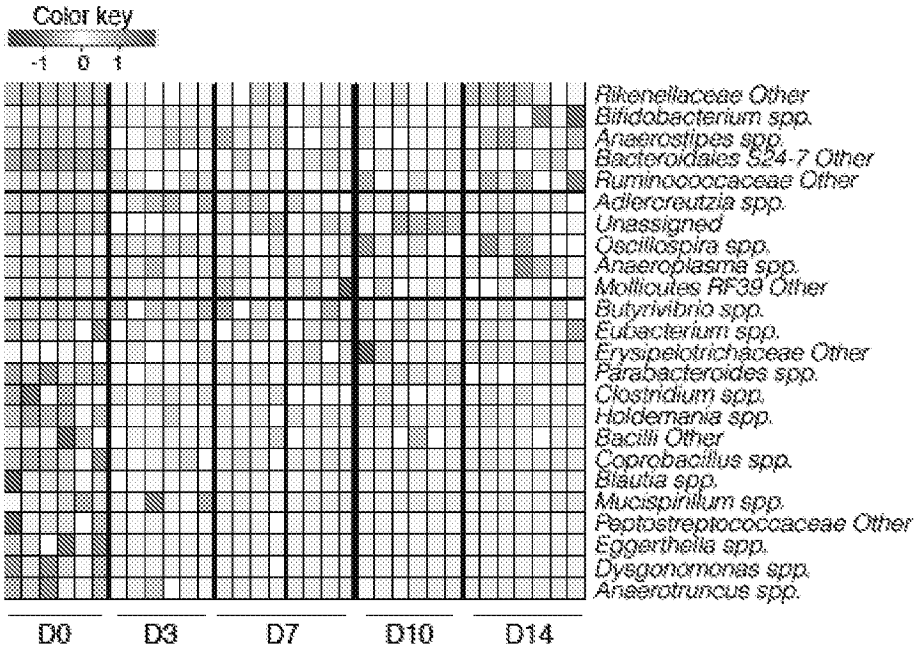

Color key
-1 0 1
Rikenellaceae Other
Bifidobacterium spp.
Anaerostipes spp.
Bacteroidales S24-7 Other
Ruminococcaceae Other
Adlercreutzia spp.
Unassigned
Oscillospira spp.
Anaeroplasma spp.
Mollicutes RF39 Other
Butyrivibrio spp.
Eubacterium spp.
Erysipelotrichaceae Other
Parabacteroides spp.
Clostridium spp.
Holdemania spp.
Bacilli Other
Coprobacillus spp.
Blautia spp.
Mucispirillum spp.
Peptostreptococcaceae Other
Eggerthella spp.
Dysgonomonas spp.
Anaerotruncus spp.
D0
D3
D7
D10
D14

FIG. 8D

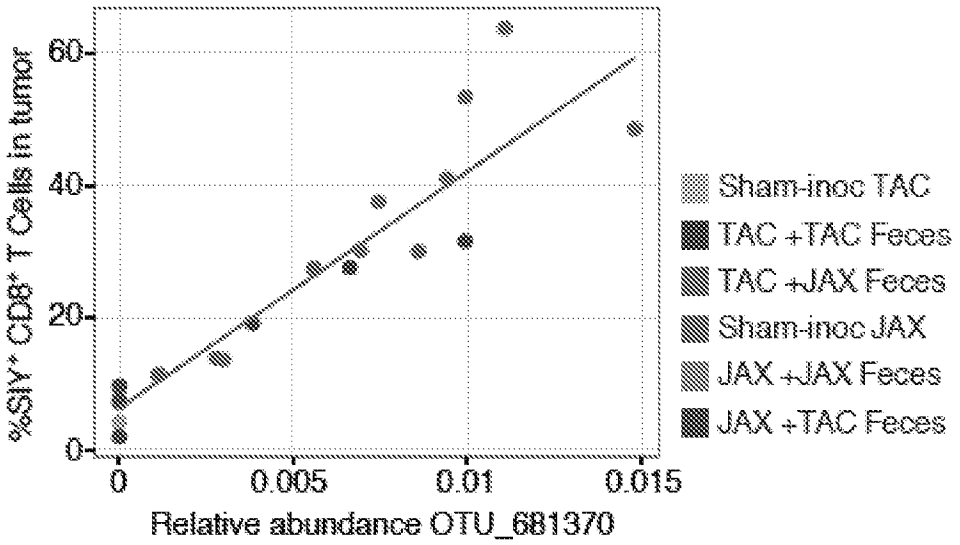

%SIY+ CD8+ T Cells in tumor
60
40
20
0
0
0.005
0.01
0.015
Relative abundance OTU_681370
Sham-inoc TAC
TAC +TAC Feces
TAC +JAX Feces
Sham-inoc JAX
JAX +JAX Feces
JAX +TAC Feces

FIG. 8E

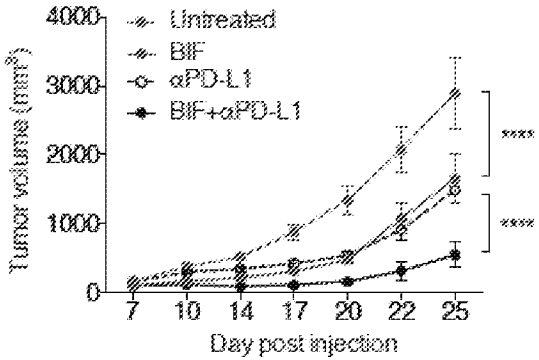


FIG. 8F

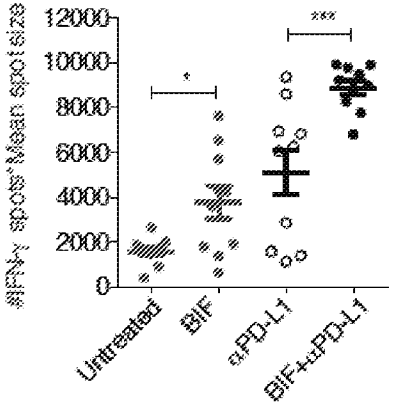


FIG. 8G

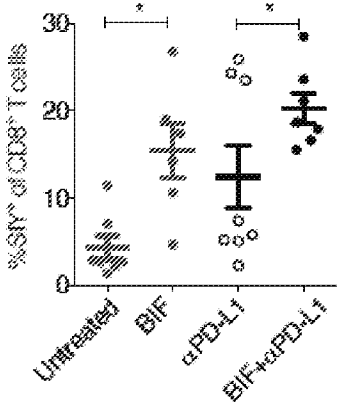


FIG. 9A

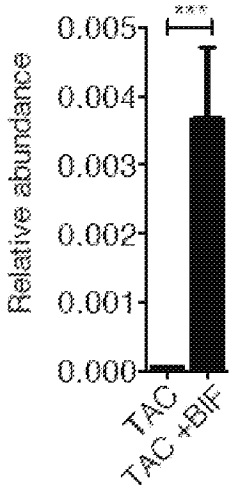


FIG. 9B

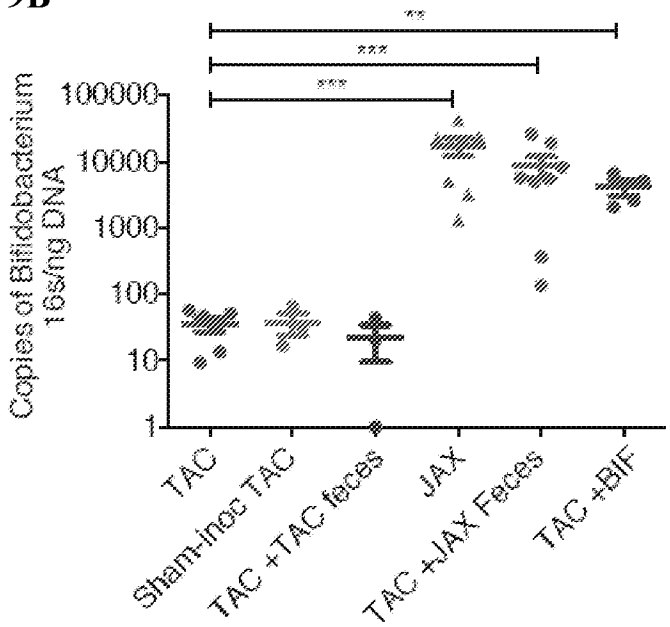


FIG. 9D

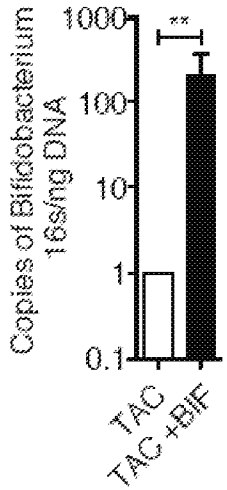


FIG. 9C

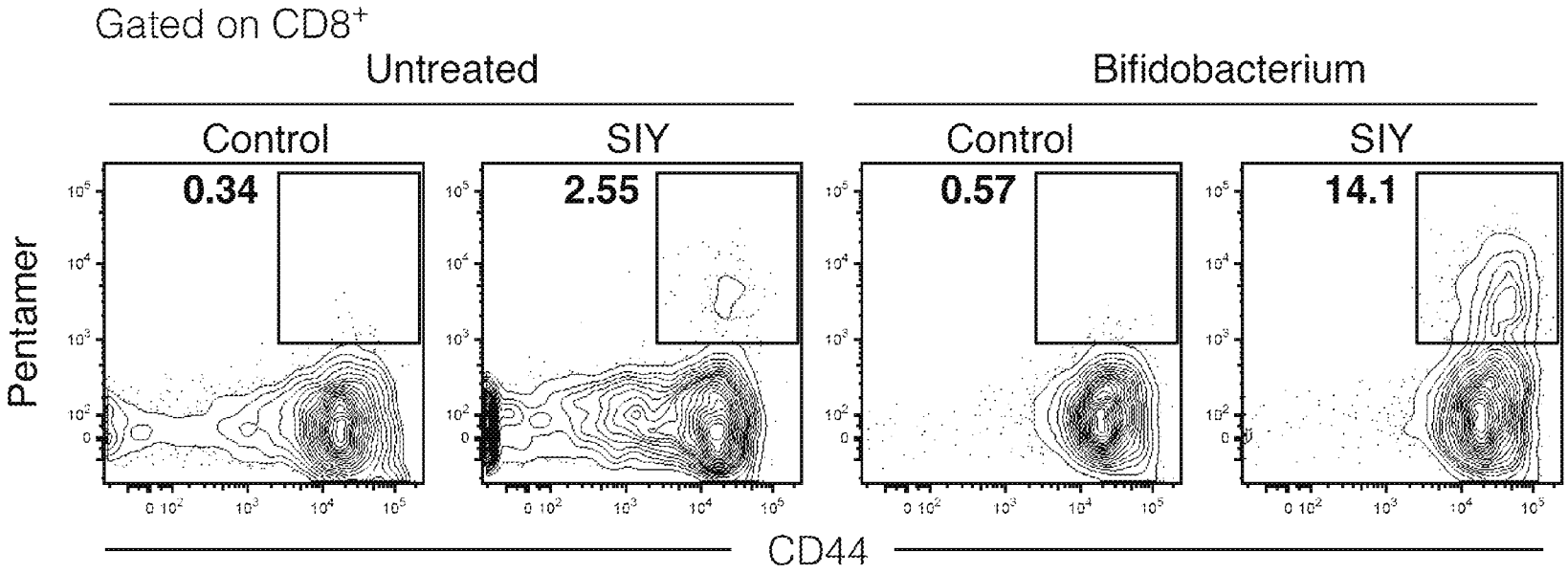

Gated on CD8+
Untreated
Bifidobacterium
Control
SIY
Control
SIY
0.34
2.55
0.57
14.1
Pentamer
CD44

FIG. 9E

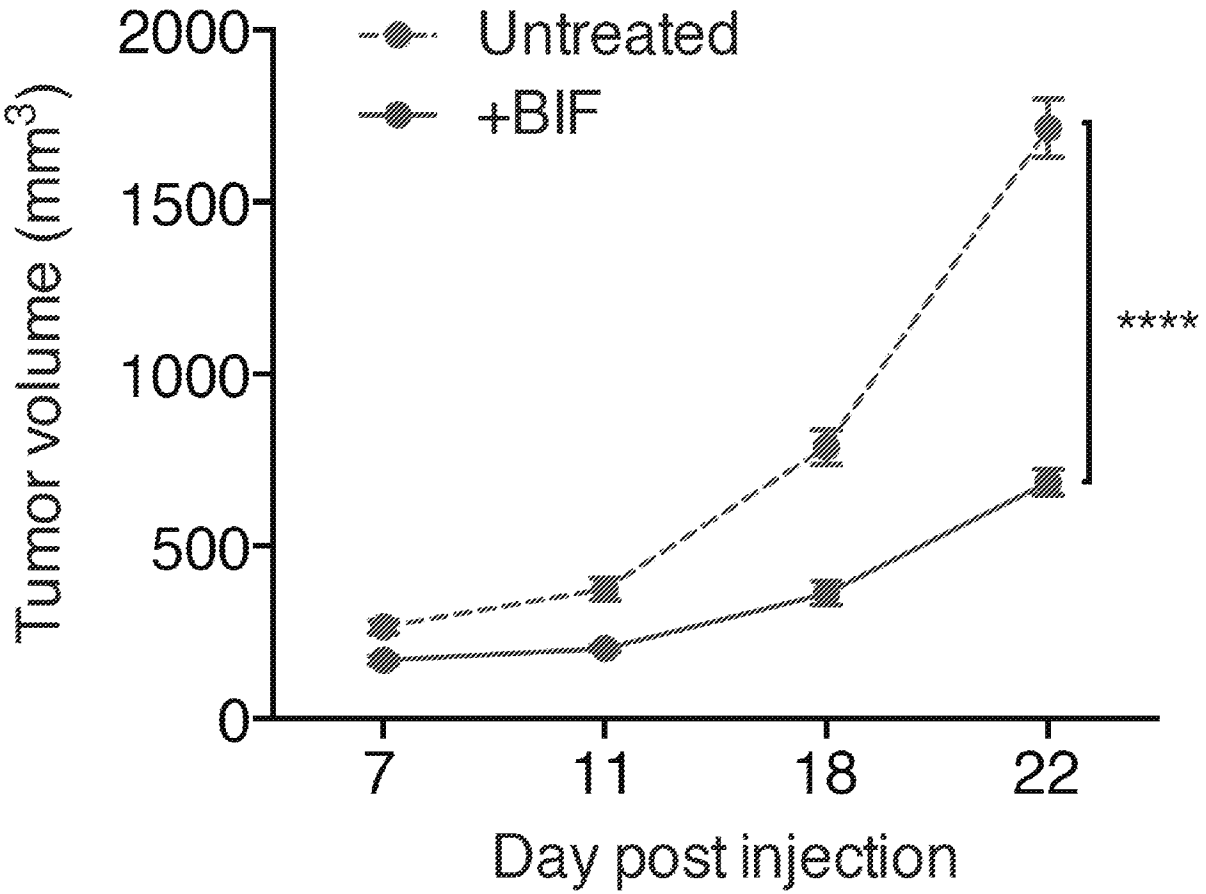

2000
1500
1000
500
0
Tumor volume (mm3)
Untreated
+BIF
****
7
11
18
22
Day post injection

FIG. 10A

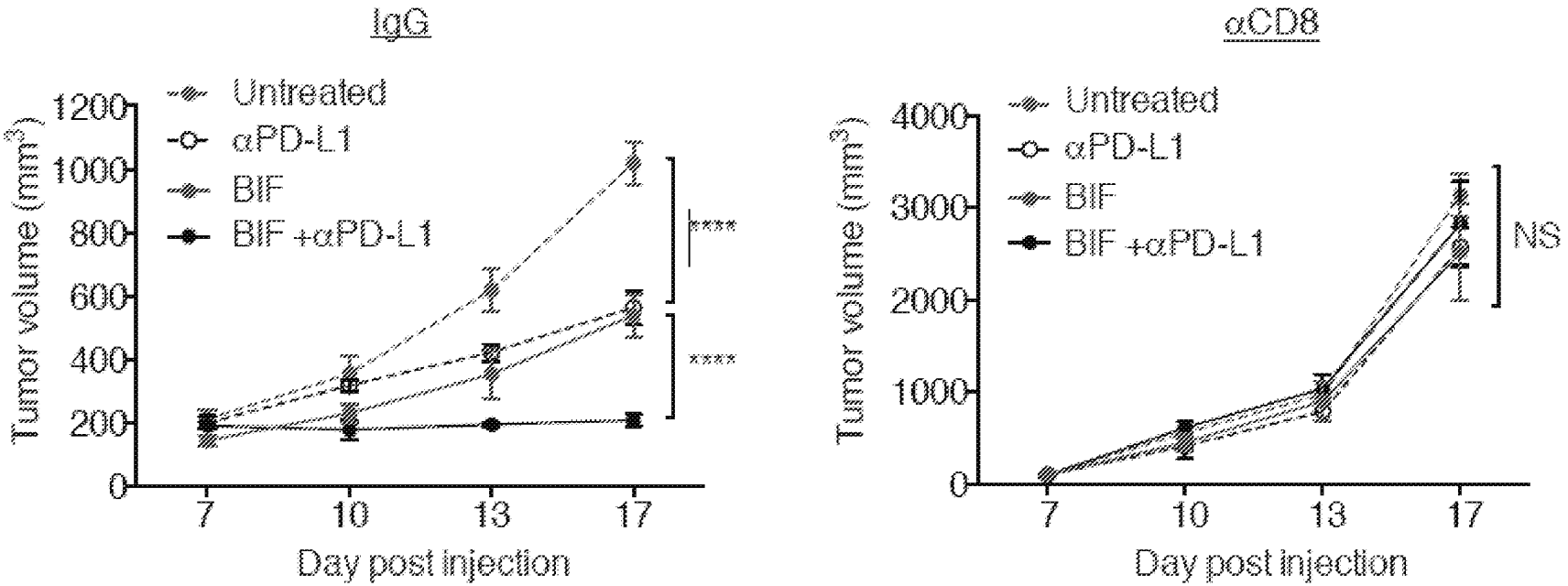

IgG
Untreated
αPD-L1
BIF
BIF +αPD-L1
Tumor volume (mm3)
1200
1000
800
600
400
200
0
7
10
13
17
Day post injection
****
****
αCD8
Untreated
αPD-L1
BIF
BIF +αPD-L1
Tumor volume (mm3)
4000
3000
2000
1000
0
7
10
13
17
Day post injection
NS

FIG. 10B

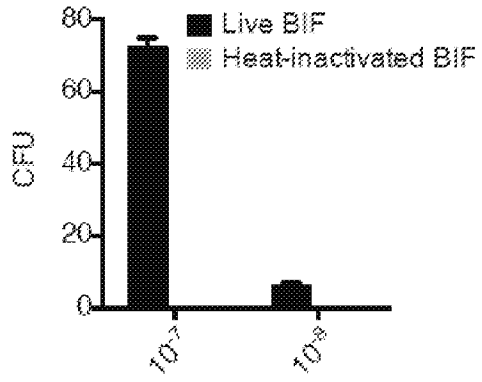


FIG. 10C

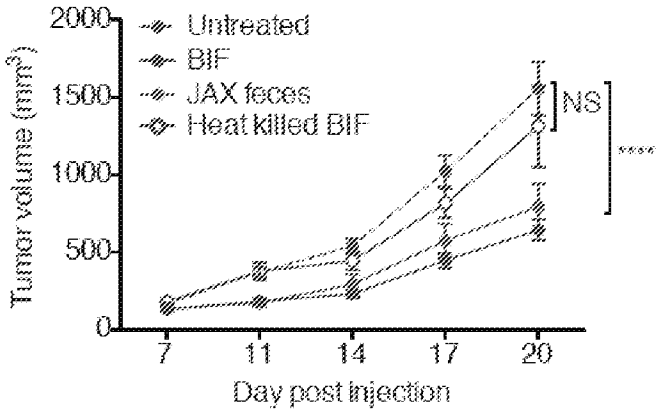


FIG. 10D

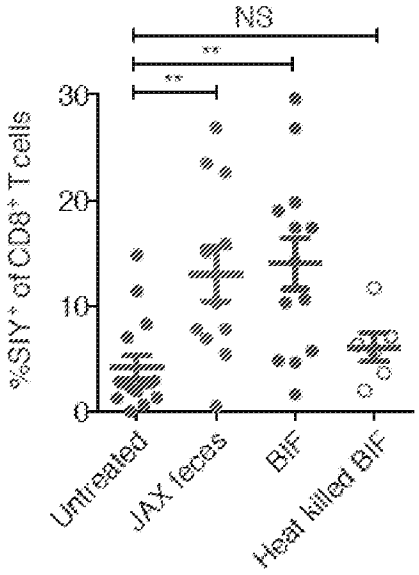


FIG. 11A

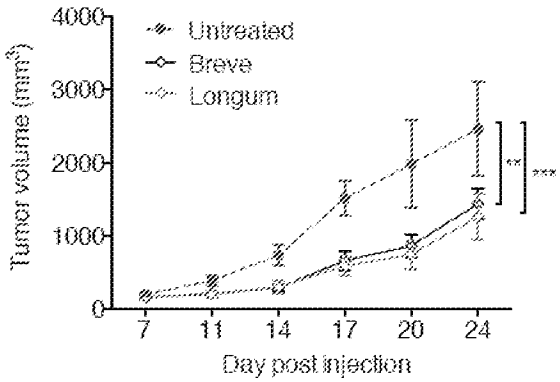


FIG. 11B

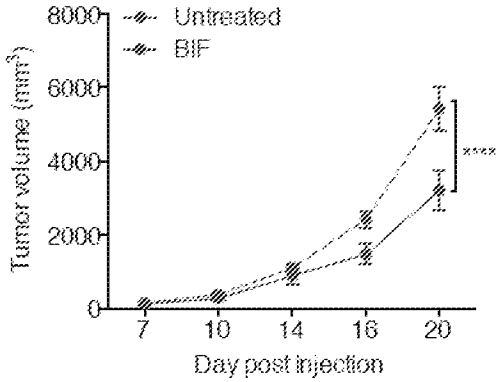


FIG. 11C

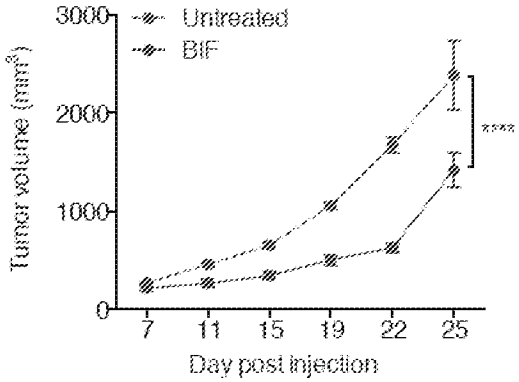


FIG. 11D

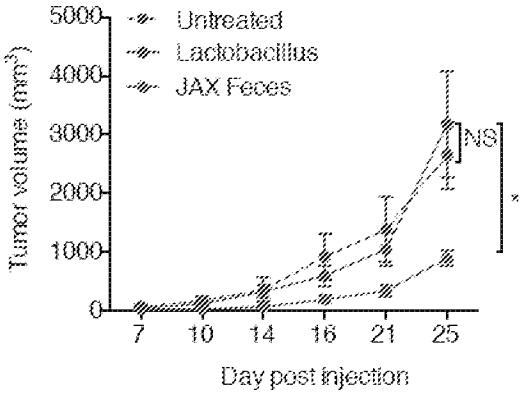


FIG. 11E

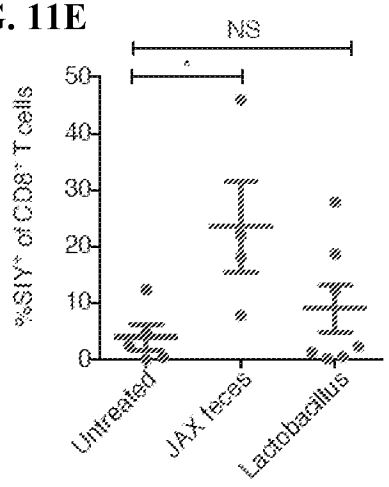


FIG. 12A

FIG. 12B

FIG. 12C

FIG. 13A

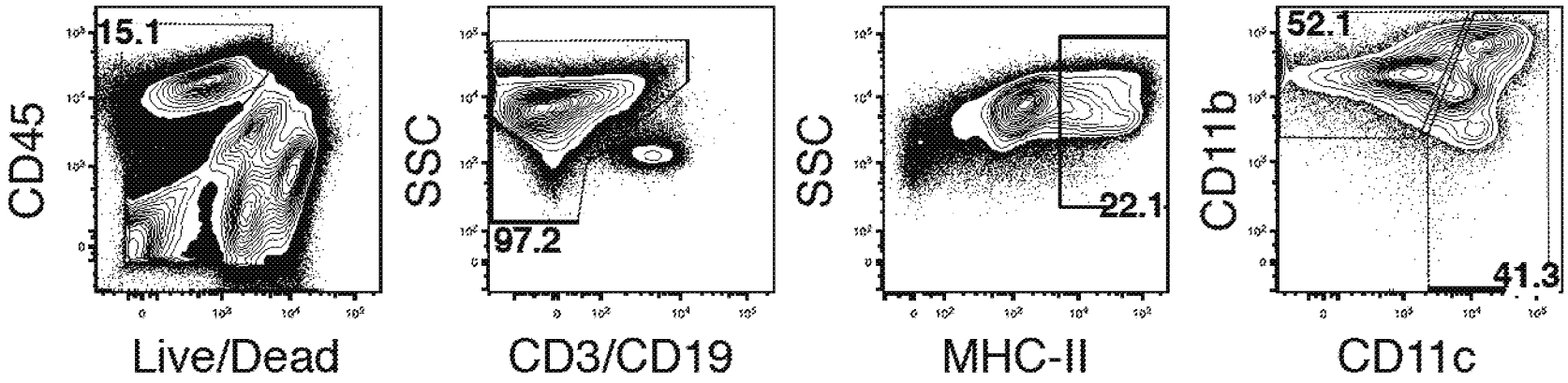

15.1
CD45
Live/Dead
SSC
97.2
CD3/CD19
SSC
22.1
MHC-II
CD11b
52.1
41.3
CD11c

FIG. 13B

fat cell differentiation
blood vessel development
protein modification by small protein conjugation
regulation of transcription, DNA-templated
regulation of apoptotic process
Ras protein signal transduction
cell activation
intracellular signal transduction
T cell mediated immunity
lymphocyte activation
small GTPase mediated signal transduction
cellular macromolecule catabolism
macromolecule catabolism
cofactor biosynthesis
cofactor metabolism
vesicle-mediated transport
negative regulation of cellular component organization
establishment of vesicle localization
intracellular transport
positive regulation of protein transport
cell cycle process
cell cycle
regulation of cell proliferation
meiotic cell cycle
mitotic cell cycle
nuclear division
organelle fission

FIG. 13C

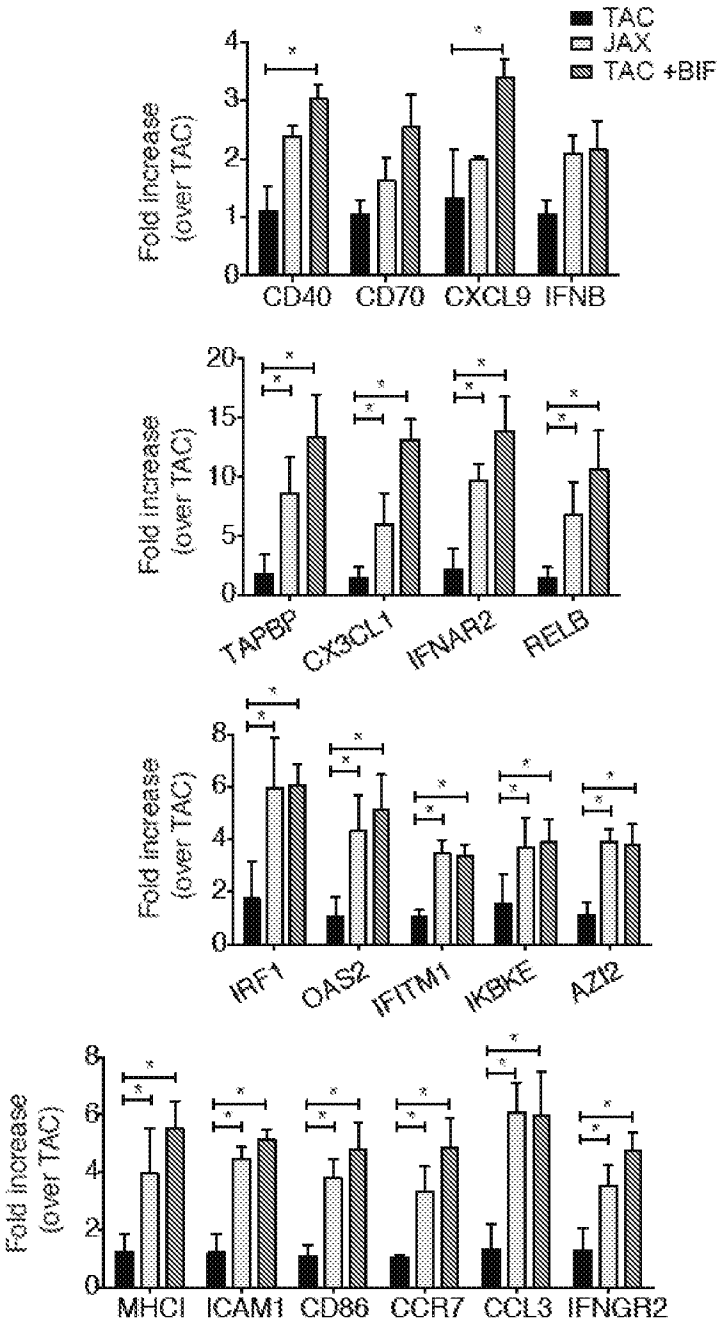

TAC
JAX
TAC +BIF
Fold increase (over TAC)
CD40
CD70
CXCL9
IFNB
TAPBP
CX3CL1
IFNAR2
RELB
IRF1
OAS2
IFITM1
IKBKE
AZI2
MHCI
ICAM1
CD86
CCR7
CCL3
IFNGR2

FIG. 14A

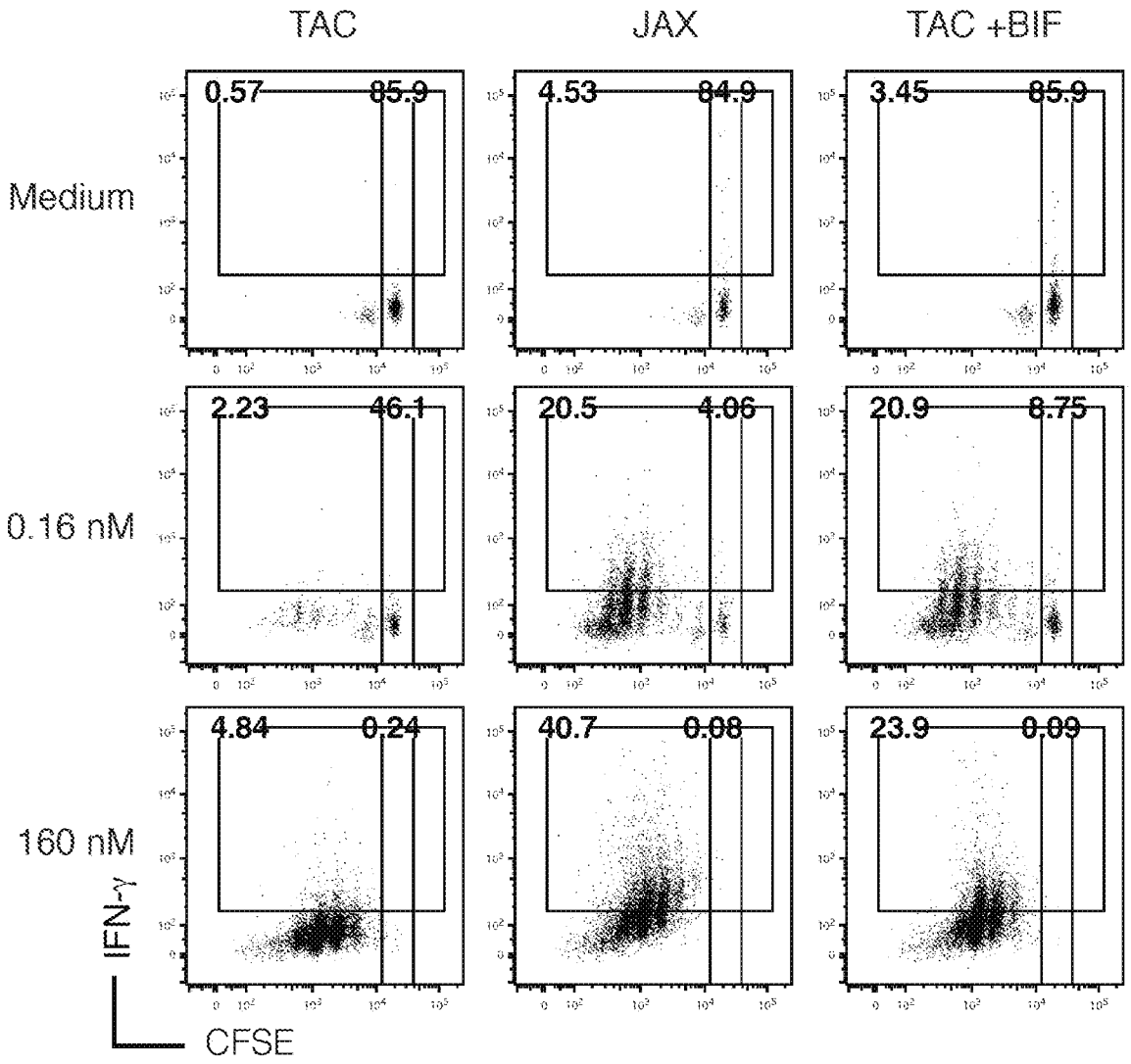

TAC
JAX
TAC +BIF
Medium
0.16 nM
160 nM
0.57
85.9
4.53
84.9
3.45
85.9
2.23
46.1
20.5
4.06
20.9
8.75
4.84
0.24
40.7
0.08
23.9
0.09
IFN-γ
CFSE

FIG. 14B

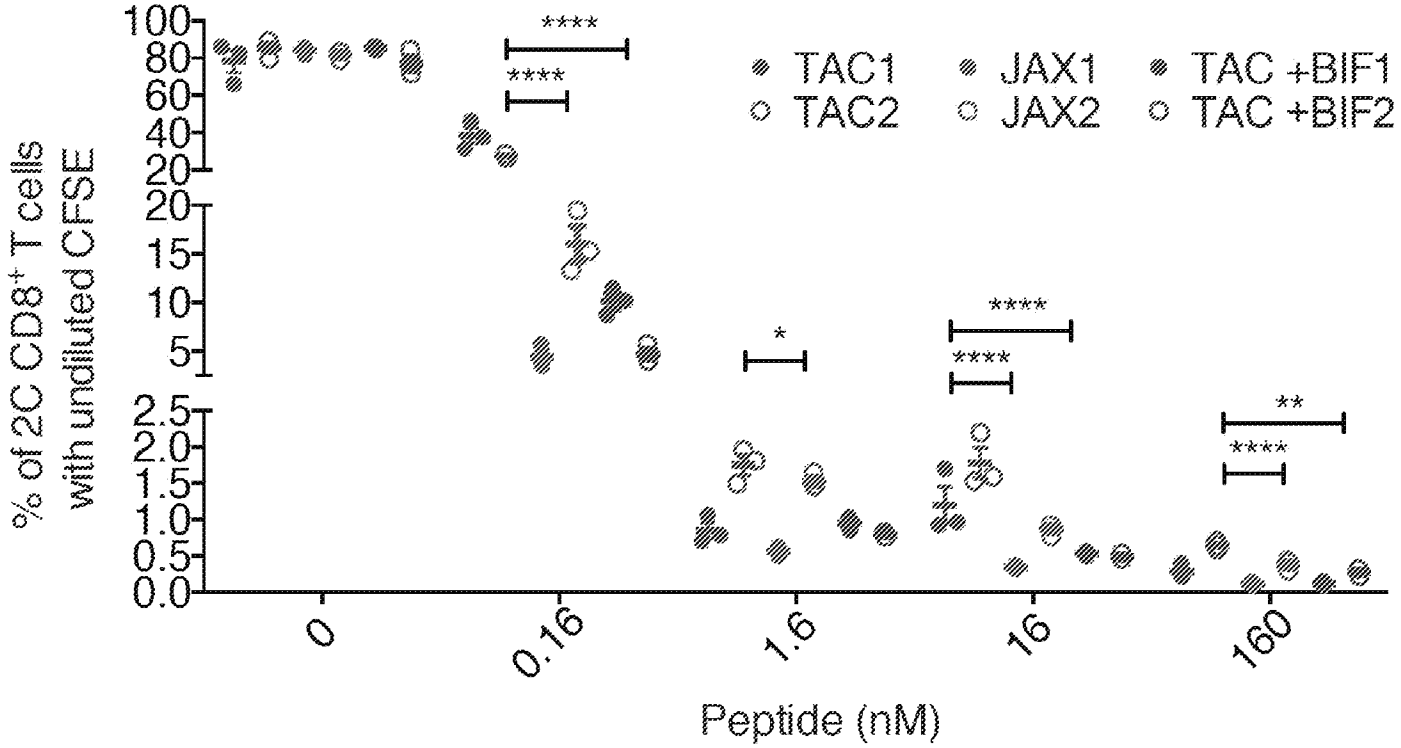
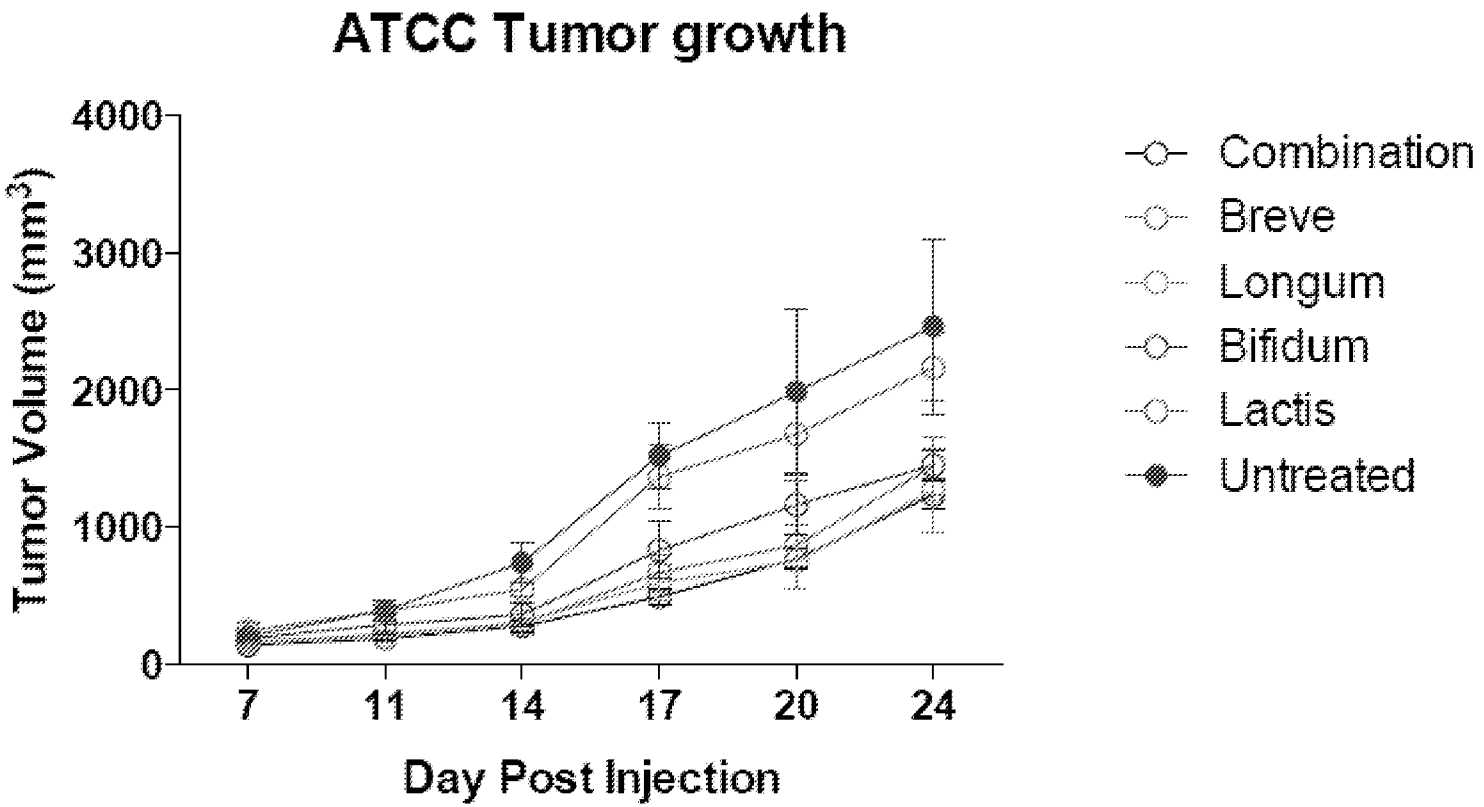

% of 2C CD8+ T cells with undiluted CFSE
100
80
60
40
20
20
15
10
5
2.5
2.0
1.5
1.0
0.5
0.0
****
****
*
****
****
**
****
TAC1
TAC2
JAX1
JAX2
TAC +BIF1
TAC +BIF2
0
0.16
1.6
16
160
Peptide (nM)

FIG. 15


ATCC Tumor growth
Tumor Volume (mm3)
4000
3000
2000
1000
0
7
11
14
17
20
24
Day Post Injection
Combination
Breve
Longum
Bifidum
Lactis
Untreated

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA
First Named Inventor/Applicant Name:	Thomas F. Gajewski
Filer:	David William Staple
Attorney Docket Number:	UCHI-34458/US-3/ORD

Filed as Small Entity

Filing Fees for Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility filing Fee (Electronic filing)	4011	1	70	70
Utility Search Fee	2111	1	300	300
Utility Examination Fee	2311	1	360	360
Request for Prioritized Examination	2817	1	2000	2000
Pages:				
Claims:				
Claims in excess of 20	2202	10	40	400
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Publ. Fee- Early, Voluntary, or Normal	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	2830	1	70	70
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**3200**

Electronic Acknowledgement Receipt

EFS ID:	25928832
Application Number:	15170284
International Application Number:	
Confirmation Number:	8885
Title of Invention:	TREATMENT OF CANCER BY MANIPULATION OF COMMENSAL MICROFLORA
First Named Inventor/Applicant Name:	Thomas F. Gajewski
Customer Number:	72960
Filer:	David William Staple/Lisa M. Day
Filer Authorized By:	David William Staple
Attorney Docket Number:	UCHI-34458/US-3/ORD
Receipt Date:	01-JUN-2016
Filing Date:	
Time Stamp:	15:34:11
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$3200
RAM confirmation Number	2265
Deposit Account	504302
Authorized User	Staple, David

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	TrackOne Request	34458US3ORD_TRACK1.pdf	124855 390faabcb59ae9d0cf87d34bf317ae87e3ae6fc0	no	2
Warnings:					
Information:					
2	Application Data Sheet	34458US3ORD_ADS.pdf	1793217 582554c6354f5b2c1a32f4ac807bceb9704c8f19	no	9
Warnings:					
Information:					
3		34458US3ORD_Application.pdf	372387 91d0b377c1918112a4b5477b58934bb2d98bda1e	yes	62

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Specification	1	57
Claims	58	61
Abstract	62	62

Warnings:					
Information:					
4	Drawings-other than black and white line drawings	34458-ORD-FIGURES.pdf	3308006 05cc5d169790e0f45f84ed07fd2e958baa9c30e4	no	38
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	42405 e98dfa0eedb5bf9bbd59d0b14bae0d0df9142789	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	5640870	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.