IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Janelle J. Bruinsma	Confirmation No.:	9571
Serial No.:	14/145,082	Group No.:	1637
Filed:	12/31/2013	Examiner:	Aaron Priest
Entitled:	**ISOLATION OF NUCLEIC ACID**		

AMENDMENT UNDER 37 C.F.R. §1.116 AFTER FINAL OFFICE ACTION MAILED JULY 6, 2015 AND REQUEST FOR AFTER-FINAL CONSIDERATION UNDER AFCP 2.0

VIA EFS WEB
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

CERTIFICATE OF EFS WEB TRANSMISSION UNDER 37 C.F.R. § 1.8

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being transmitted to the United States Patent and Trademark Office transmitted via the Office electronic filing system in accordance with 37 C.F.R. §1.6(a)(4).

Dated: July 31, 2015 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This communication is an After Final Amendment under 37 C.F.R. §1.116, responsive to the Final Office Communication mailed July 6, 2015, with a response due on or before October 6, 2015, and is filed with a Request for After-Final Consideration under AFCP 2.0. Applicants respectfully request entry of this amendment.

The Commissioner is hereby authorized 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.: EXCT-31920/US-3/CON. 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 Claims are reflected in the Listing of Claims beginning on page 2.

Remarks begin on page 5.

AMENDMENTS TO THE CLAIMS:

This listing of the claims will replace all prior listings and versions of claims in the application:

1. (currently amended) A system for isolating a target human DNA from a human stool sample, the system comprising:

a) a volume of stool homogenization solution suitable for processing a human stool sample having a mass of at least 4 grams;

b) an inhibitor-adsorbing composition comprising insoluble polyvinylpolypyrrolidone particles;

c) a spin filter, comprising

i) a hollow body;

ii) a bottom end; and

iii) an open top end opposite the bottom end,

wherein the hollow body of said spin filter is made from a porous filtering material;

d) guanidine thiocyanate; and

e) ~~a magnetic target-specific capture particle comprising a covalently-attached oligonucleotide that is complementary to at least a portion of a target human DNA selected from the group consisting of *vimentin*, *NDRG4*, *BMP3*, *TFPI2* and *beta-actin*.~~ a plurality of different magnetic target-specific capture particles, wherein different target-specific capture particles comprise covalently-attached oligonucleotides complementary to at least a portion of different target human DNAs, and wherein said different target human DNAs comprise *NDRG4*, *BMP3*, and *KRAS*.

2. (original) The system of claim 1, further comprising a magnet.

3-4. (cancelled)

5. (previously presented) The system of claim 1 wherein said polyvinylpolypyrrolidone is in a premeasured form.

6. (previously presented) The system of claim 3 wherein said polyvinylpolypyrrolidone is provided as a capsule or pressed tablet comprising a premeasured amount of polyvinylpolypyrrolidone.

7. (cancelled)

8. (previously presented) The system of claim 1, further comprising an elution solution and/or a wash solution.

9-12. (cancelled)

13. (previously presented) The system of claim 1, further comprising a sample container configured to hold said stool sample.

14. (original) The system of claim 1 further comprising a vessel in which to hold isolated target human DNA.

15. (original) The system of claim 1, further comprising a shipping container.

16. (original) The system of claim 1, further comprising a control reagent.

17. (original) The system of claim 16, wherein said control comprises a nucleic acid.

18-23. (cancelled)

24. (currently amended) The system of claim 1, wherein said covalently-attached oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NOS:~~1–5~~2-3 and 6-7.

25. (previously presented) The system of claim 1, further comprising a magnetic target-specific capture particle comprising an oligonucleotide that is complementary to at least a portion of a human reference DNA.

26. (currently amended) The system of claim 1, ~~comprising a plurality of different magnetic target-specific capture particles, wherein different target-specific capture particles comprise covalently-attached oligonucleotides complementary to at least a portion of different target human DNAs, and~~ wherein said ~~different target human DNAs comprise a~~ plurality of target human DNAs ~~selected from the group consisting of~~ further comprises *vimentin*~~, *NDRG4*, *BMP3*~~, *TFPI2*, *or beta-actin*~~, and *KRAS*~~.

27. (previously presented) The system of claim 26, wherein said covalently-attached oligonucleotides comprise sequences selected from the group consisting of SEQ ID NOS:1-7.

28. (currently amended) The system of claim ~~26~~25, wherein said ~~plurality of different target human DNAs comprises *vimentin*, *NDRG4*, *BMP3*, *TFPI2*,~~human reference DNA is *beta-actin*~~, and *KRAS*~~.

29. (currently amended) The system of claim 28, wherein said covalently-attached oligonucleotides comprise the ~~sequences of SEQ ID NOS:1-7~~sequence of SEQ ID NO:5.

REMARKS

Claims 1-2, 5-6, 8, 13-17 and 24-29 are pending and under examination in the present application. In the Office Action mailed July 6, 2015, the Examiner made the following objections and rejections:

Claims 1-2, 5, 8, 13-14, 16-17 and 24-29 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over BAKER (US 2003/0173284, published 9/18/2003), in view of, SHUBER (US 2002/0164631, published 11/7/2002) and FOTEDAR et al. (Laboratory Diagnostic Techniques for Entamoeba Species, Clinical Microbiology Reviews, July 2007, p. 511-532; as evidenced by MORGAN et al. (Comparison of PCR and Microscopy for Detection of Cryptosporidium parvum in Human Fecal Specimens: Clinical Trial, J. Clin Micro, Apr. 1998, p. 995-998), in further view of VAN ENGELAND (WO 2008084219, published 7/17/2008), ZOU, et al. (Highly Methylated Genes in Colorectal Neoplasia: Implications for Screening, Cancer Epidemiol Biomarkers Prevo 2007 Dec;16(12):2686-96.), NCBI ACCESSION NOs. X56134 (10/7/2008), AC009118 (3/18/2003), NM_006528 (5/2/2010), NG_007992 (5/7/2010), NM_001201 (3/5/2010) and NM_033360 (4/25/2010).

Claim 6 is rejected under 35 U.S.C. § 103(a) as being unpatentable over BAKER, in view of SHUBER and FOTEDAR (as evidenced by MORGAN), in further view of VAN ENGELAND, ZOU, NCBI ACCESSION NOs. X56134 (10/7/2008), AC009118 (3/18/2003), NM_006528 (5/2/2010), NG_007992 (5nt2010), NM_001201 (3/5/2010) and NM_033360 (4/25/2010), in further view of Qiagen (QIAamp DNA Stool Mini Kit Handbook, 8/2001).

Claim 15 is rejected under 35 U.S.C. § 103(a) as being unpatentable over BAKER, in view of SHUBER and FOTEDAR (as evidenced by MORGAN), in further view of VAN ENGELAND, ZOU, NCBI ACCESSION NOs. X56134 (10/7/2008), AC009118 (3/18/2003), NM_006528 (5/2/2010), NG_007992 (5nt2010), NM_001201 (3/5/2010) and NM_033360 (4/25/2010), in further view of STEPHAN (US 2010/0293130, published 11/18/2010, effective filing date 11/30/2006).

Applicants note with appreciation that the Examiner has suggested amendment to include all of the genes shown in the multi-marker stool test of Example 7. However, for the reasons discussed below, for business reasons and to advance prosecution, and reserving the right to prosecute the original or similar claims in one or more future applications, Applicants herein

amend Claim 1 to recite " . . . a plurality of different magnetic target-specific capture particles, wherein different target-specific capture particles comprise covalently-attached oligonucleotides complementary to at least a portion of different target human DNAs, and wherein said different target human DNAs comprise *NDRG4*, *BMP3*, and *KRAS*".

The claims are not obvious

The Examiner asserts that all of vimentin, NDRG4, BMP3, TFPI2, and KRAS were known colorectal cancer markers, and beta-actin was a familiar control gene at the time of the invention, and that a skilled artisan at the time of the invention would have had an interest in all of the above genes in order to detect colorectal cancer.

Applicants respectfully submit that the cited art does not teach or suggest selection of the particular combination of 'genes of interest' recited in the claims as amended, nor the particular combination of reagents used to assemble a sufficiently successful stool DNA assay for the detection of colorectal cancer. In contrast, Applicants have now shown that the particular combination of target human DNAs and system components for capturing them provide an the basis for an assay that has received regulatory approval. Specifically, the system components of instant claim 1 as amended are included in Applicants' product, the "Cologuard®" colorectal cancer screening test, which is the first stool DNA screening test for colorectal cancer to receive FDA approval. The cited art does not teach or suggest the *particular* combination of NDRG4, BMP3 and KRAS target human DNAs, or the collected set of system components to perform the steps to isolate these particular target human DNAs from human stool samples.

Provided herewith are the following documents:

1. FDA Press Release "FDA approves first non-invasive DNA screening test for colorectal cancer." August 8, 2014; announcing approval of the "Cologuard®" stool DNA test product;
2. A set of instructions for the commercial assay entitled "Cologuard® sDNA-based Colorectal Cancer Screening Test Instructions for Use" ("Instructions") (a draft version of which is also available for download on the World Wide Web at fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/MedicalDevices/

MedicalDevicesAdvisoryCommittee/MolecularandClinicalGeneticsPanel/UCM390231.pdf), providing a complete description of the FDA-approved screening test.

As stated in the second paragraph of the "Principles of the Procedure" beginning on page 8, the target human DNAs are NDRG4, BMP3, KRAS, and a reference gene (beta actin, ACTB):

> In the processing procedure for DNA testing, the stool sample is mixed with buffer in the Container using the Sample Mixer. An aliquot of the buffered stool sample is centrifuged to pellet solids and generate supernatant. The assay procedure begins with treatment of the supernatant with an Inhibitor Removal Tablet to remove inhibitors that may affect the detection of the DNA biomarkers. Treated supernatant is then combined with denaturing reagents and incubated with target-specific magnetic particles using the Capture Incubator instrument to capture sequences for *NDRG4*, *BMP3*, *KRAS*, and *ACTB* (reference gene).

(Instructions for Use, page 8)

This document further shows that the FDA-approved test comprises the system components recited in the instant claims as amended: stool homogenization buffer, PVPP inhibitor adsorbant, spin filter and guanidine thiocyanate (see Instructions, page 11, items 200204, 200151, 200138, and 200121, respectively), and magnetic target-specific capture particles for capturing NDRG4, BMP3 and KRAS target human DNAs ("Capture Beads", Instructions, page 9, item 200150).

3. Thomas F. Imperiale, M.D., et al., "Multitarget Stool DNA Testing for Colorectal-Cancer Screening", New England Journal of Medicine 370(14):1287 (2014).

This document reports on the clinical trial of the Cologuard® test. Page 6 of the Appendix, "Supplemental Figure Legend" for Figure S1 shows that the method used in the study comprised use of system components according to instant claim 1 as amended:

100 ng Hb / ml buffer. The hemoglobin component of the multi-target sDNA test was analyzed by a separate immunochemical assay. The buffered residual stool sample was normalized with additional buffer to a 1 gm:5 ml stool buffer ratio and homogenized. Homogenates were aliquoted 42 ml/tube and frozen at -80°C until analyzed. Aliquots were thawed at room temperature, centrifuged and the supernatant was treated with polyvinylpyrrolidone to remove PCR inhibitors and spin filtered to clarify. Sequence specific DNA biomarker targets were isolated directly from the clarified supernatant using a magnetic bead based oligonucleotide hybrid capture method. The captured DNA was split and one portion was subjected to the bisulfite reaction for the identification of aberrantly methylated *NDRG4* and *BMP3* and the other portion was used to determine the presence of any of seven *K-ras* point mutations. The test does not distinguish the specific mutation. Using quantitative real-time target and signal

(Imperiale, et al., Supplementary Appendix, page 6)

The data published by Imperiale show that:

- Sensitivity of the Cologuard assay in detecting patients with colorectal cancer was 92% versus 74% for FIT (fecal immunochemical test) ;
- Sensitivity in detecting patients with colorectal cancers in Stages I-III —those determined by the American Joint Committee on Cancer to be associated with an increased rate of being cured—was 93% for Cologuard versus 73 % for FIT;
- Sensitivity for patients with advanced pre-cancerous lesions was 42% for Cologuard versus 24% for FIT;
- Cologuard detected 69% of patients with polyps with high-grade dysplasia versus 46% for FIT;
- Cologuard achieved a specificity of 87% versus FIT specificity of 95%.

(Imperiale, page 1293, col 1 and Table 1).

Long felt need and the failure of others

As evidenced by the art cited by the Examiner, isolation of human DNA from stool samples for use in colorectal cancer screening has long been a goal in this field (see, *e.g.*, SHUBER as cited above, published in 2002; ZOU and VAN ENGELAND, published in 2007 and 2008, respectively), and different combinations of markers to detect colorectal cancer in stool samples have been tried (see, *e.g.*, ZOU and VAN ENGELAND).

VAN ENGELAND provides a detailed summary of the state of the art of isolating DNA from stool samples in 2008, and highlights that these methods are unsatisfactory:

> The initial DNA isolation techniques typically recovered DNA from 10g to 4g stool and more conveniently purified the human DNA component using streptavidin-bound magnetic beads (Dong et al., 2001; Ahlquist et al., 2000). Further improvements in recovery of target human DNA from stool comprised an electrophoresis-driven separation of target DNA sequences, using oligonucleotide capture probes immobilised in an acrylamide gel (Whitney et al., 2004). Later, when DNA integrity proved to be a suitable marker it was also important to prevent degradation during sample handling. Improved results were obtained with stool samples frozen as quickly as possible after collection. Alternatively, stabilization buffer was added to the stool samples before further transport (Olson et al., 2005). A recent improvement involves the use of an MBD column to extract methylated human DNA in a high background of fecal bacterial DNA (Zou et al., 2007). However, despite these advances, current tools for cancer detection in faecal samples are still unsatisfactory.

(VAN ENGELAND, page 6, line 21 to page 7, line 7, emphasis added)

The references cited in making the rejection, including SHUBER, ZOU, and VAN ENGELAND, demonstrate that there has been a long-felt need for a way to screen for colorectal cancers based on stool DNA testing. However, even in possession of the methods and markers disclosed in the art cited by the Examiner, as stated by VAN ENGELAND, other workers in the field have failed. In contrast, Applicants have been able to meet this need and succeed where others had failed by using a particular combination of markers and methods for extracting the marker DNA, as described in the instant application. The embodiments as claimed herein addressed this long-felt need and succeeded where others had failed, as evidenced by first-ever FDA approval for the product containing these components.

Accordingly, it is respectfully submitted that the claims are not obvious and the rejection is overcome, and Applicants respectfully request that the rejection be withdrawn.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that all objections and rejections have been addressed, and Applicants' claims should be passed to allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicants encourage the Examiner to call the undersigned collect at (608) 662-1277.

Dated: July 31, 2015

/Mary Ann D. Brow/

Mary Ann D. Brow
Registration No. 42,363

CASIMIR JONES, S.C.
2275 Deming Way, Suite 310
Middleton, Wisconsin 53562
608.662.1277

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Multitarget Stool DNA Testing for Colorectal-Cancer Screening

Thomas F. Imperiale, M.D., David F. Ransohoff, M.D., Steven H. Itzkowitz, M.D., Theodore R. Levin, M.D., Philip Lavin, Ph.D., Graham P. Lidgard, Ph.D., David A. Ahlquist, M.D., and Barry M. Berger, M.D.

ABSTRACT

BACKGROUND

An accurate, noninvasive test could improve the effectiveness of colorectal-cancer screening.

METHODS

We compared a noninvasive, multitarget stool DNA test with a fecal immunochemical test (FIT) in persons at average risk for colorectal cancer. The DNA test includes quantitative molecular assays for *KRAS* mutations, aberrant *NDRG4* and *BMP3* methylation, and β-actin, plus a hemoglobin immunoassay. Results were generated with the use of a logistic-regression algorithm, with values of 183 or more considered to be positive. FIT values of more than 100 ng of hemoglobin per milliliter of buffer were considered to be positive. Tests were processed independently of colonoscopic findings.

RESULTS

Of the 9989 participants who could be evaluated, 65 (0.7%) had colorectal cancer and 757 (7.6%) had advanced precancerous lesions (advanced adenomas or sessile serrated polyps measuring ≥1 cm in the greatest dimension) on colonoscopy. The sensitivity for detecting colorectal cancer was 92.3% with DNA testing and 73.8% with FIT (P=0.002). The sensitivity for detecting advanced precancerous lesions was 42.4% with DNA testing and 23.8% with FIT (P<0.001). The rate of detection of polyps with high-grade dysplasia was 69.2% with DNA testing and 46.2% with FIT (P=0.004); the rates of detection of serrated sessile polyps measuring 1 cm or more were 42.4% and 5.1%, respectively (P<0.001). Specificities with DNA testing and FIT were 86.6% and 94.9%, respectively, among participants with nonadvanced or negative findings (P<0.001) and 89.8% and 96.4%, respectively, among those with negative results on colonoscopy (P<0.001). The numbers of persons who would need to be screened to detect one cancer were 154 with colonoscopy, 166 with DNA testing, and 208 with FIT.

CONCLUSIONS

In asymptomatic persons at average risk for colorectal cancer, multitarget stool DNA testing detected significantly more cancers than did FIT but had more false positive results. (Funded by Exact Sciences; ClinicalTrials.gov number, NCT01397747.)

From the Department of Medicine, Indiana University School of Medicine, the Regenstrief Institute, the Simon Cancer Center, and the Center for Innovation at Roudebush Veterans Affairs Medical Center — all in Indianapolis (T.F.I.); the Departments of Medicine and Epidemiology and the Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill (D.F.R.); the Dr. Henry D. Janowitz Division of Gastroenterology, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York (S.H.I.); Kaiser Permanente Medical Center, Walnut Creek, CA (T.R.L.); Boston Biostatistics Research Foundation, Framingham MA (P.L.); Exact Sciences, Madison, WI (G.P.L., B.M.B.); and the Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN (D.A.A.). Address reprint requests to Dr. Imperiale at Indiana University Medical Center–Regenstrief Institute, 1050 Wishard Blvd., Indianapolis, IN 46202.

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Colorectal cancer is a major cause of death and disease among men and women in the United States.[1] The underlying neoplastic processes of colorectal carcinogenesis lend themselves to screening.[2] Evidence supports and guidelines endorse several tests and strategies,[3-5] and screening for colorectal cancer has been found to be cost-effective.[5-7]

Despite the supporting evidence, recommendations, and availability of several screening tests, a substantial proportion of the U.S. population is not up to date with screening.[8] A simple, noninvasive test with high sensitivity for both colorectal cancer and advanced precancerous lesions might increase uptake and adherence rates, which could improve clinical outcomes.

Colorectal cancer arises from accumulated genetic and epigenetic alterations, which provide a basis for the analysis of stool to identify tumor-specific changes.[9] Large-scale screening studies of previously available stool-based DNA tests showed only fair sensitivity for the detection of colorectal cancer (i.e., the capacity to detect cancers, or true positive tests [see Glossary]) and low sensitivity for the detection of advanced adenomas.[10,11] Important advances have since been incorporated, including the use of a stabilizing buffer,[12,13] more discriminating markers,[14,15] more sensitive analytic methods,[14,16,17] automation,[16] and an overall determination of results with the use of a logistic-regression algorithm, which together result in higher sensitivity for the detection of both cancer and advanced precancerous lesions.[14,16] However, evaluation of the more recent tests was based largely on analyses of archived specimens, including those collected from patients after the diagnosis but before the resection of colorectal cancer or advanced precancerous polyps.

In this study, we evaluate the multitarget stool DNA test as a tool for screening. The primary aim was to determine the performance characteristics of the DNA test in the detection of colorectal cancer. The secondary aims were to determine the performance of the DNA test in the detection of advanced precancerous lesions and to compare it with a commercially available fecal immunochemical test (FIT) for human hemoglobin in the detection of both colorectal cancer and advanced precancerous lesions.

METHODS

STUDY DESIGN

From June 2011 through November 2012, we enrolled participants in this cross-sectional study at 90 sites throughout the United States and Canada, including private-practice and academic settings. The study was approved by the institutional review board at each site, and all participants provided written informed consent.

The study, which was funded by Exact Sciences, was designed by the authors; Health Decisions, a contract research organization, gathered and monitored the data. The first author wrote the first draft of the manuscript, incorporating the other authors' contributions; one of the authors, who is a statistician, analyzed the data and, along

Glossary of Screening Terms

Sensitivity (true positive rate): The proportion of persons with disease who have a positive test (positive test results among persons with disease).

Specificity (true negative rate): The proportion of persons without disease who have a negative test (negative test results among persons without disease).

False negative rate (1 minus sensitivity): The proportion of persons with disease who have a negative test (negative test results among persons with disease).

False positive rate (1 minus specificity): The proportion of persons without disease who have a positive test (positive test results among persons without disease).

Positive predictive value: The proportion of persons with disease among those with a positive test (disease present among those with positive test results).

Negative predictive value: The proportion of persons without disease among those with a negative test (disease absent among those with negative test results).

Number needed to screen: The number of persons who would need to be screened to identify one person with the disease.

with the last author, vouches for the data and adherence to the study protocol, which is available with the full text of this article at NEJM.org. All the authors signed confidentiality agreements with Exact Sciences.

STUDY POPULATION

The target population was asymptomatic persons between the ages of 50 and 84 years who were considered to be at average risk for colorectal cancer and who were scheduled to undergo screening colonoscopy. Enrollment was weighted toward persons 65 years of age or older in order to increase the prevalence of cancer. We excluded participants who had a personal history of colorectal neoplasia, digestive cancer, or inflammatory bowel disease; had undergone colonoscopy within the previous 9 years or a barium enema, computed tomographic colonography, or sigmoidoscopy within the previous 5 years; had positive results on fecal blood testing within the previous 6 months; had undergone colorectal resection for any reason other than sigmoid diverticula; had overt rectal bleeding within the previous 30 days; had a personal or family history of colorectal cancer; had participated in any interventional clinical study within the previous 30 days; or were unable or unwilling to provide written informed consent.

CLINICAL PROCEDURES

All participants were required to provide a stool specimen and undergo screening colonoscopy within 90 days after providing informed consent. Stool was collected before routine bowel preparation. No dietary or medication restrictions were required. Colonoscopists were required to describe the extent of the examination, document cecal visualization, rate the quality of preparation (on a modified Aronchick scale),[18] and record the size and location of lesions.

Although colonoscopists reported the location and size of all lesions, only the most advanced colorectal epithelial lesion (the index lesion) and its location (proximal or distal) were used to categorize participants for the analysis. If two similarly advanced lesions were present, the larger of the two was designated as the index lesion. The proximal colon was considered to include the splenic flexure and all segments proximal to it, an insertion depth of more than 60 cm, or any part described by the phrase "right colon"; the distal colon was considered to include all other segments, an insertion depth of 60 cm or less, or any part described by the phrase "left colon."

The biopsy and surgical specimens underwent histopathological analysis at the laboratory typically used by each study site. Polyps with high-grade dysplasia or 25% or more villous elements in adenomas measuring less than 1 cm, as well as sessile serrated or hyperplastic polyps measuring 1 cm or larger, were re-reviewed centrally by a gastrointestinal pathologist for confirmation, with diagnostic disagreements resolved by consensus of at least two central pathologists.

PRIMARY AND SECONDARY OUTCOMES

The primary outcome was the ability of the DNA test to detect colorectal cancer (i.e., adenocarcinoma), with disease stage determined with the use of the American Joint Committee on Cancer (AJCC) staging system.[19] The secondary outcome was the performance of the DNA test for the detection of advanced precancerous lesions, including advanced adenomas (high-grade dysplasia or with ≥25% villous histologic features or measuring ≥1 cm in the greatest dimension) and sessile serrated polyps measuring 1 cm or more in diameter.

LABORATORY PROCEDURES

A central biorepository received all stool specimens. Laboratory testing was performed without knowledge of the results of either the comparator FIT or clinical findings. (Details of stool collection and processing for DNA testing are shown in Fig. S1 in the Supplementary Appendix, available at NEJM.org.) Buffered stool samples were homogenized, separated into aliquots, and frozen at −80°C on receipt. Stool aliquots were subsequently sent in batches to one of three laboratories: Exact Sciences (Madison, WI), Mayo Medical Laboratory (Rochester, MN), and Molecular Pathology Laboratory Network (Knoxville, TN). Each laboratory received, in a blinded fashion, a similar distribution of specimens on the basis of colonoscopic findings.

The multitarget stool DNA test consists of molecular assays for aberrantly methylated *BMP3* and *NDRG4* promoter regions, mutant *KRAS*, and β-actin (a reference gene for human DNA quantity), as well as an immunochemical assay for

human hemoglobin. Quantitative measurements of each marker were incorporated into a validated, prespecified logistic-regression algorithm, with a value of 183 or more indicating that the test result was positive (for details, see the Supplementary Appendix). Analytic results were transferred to the study's biostatistician.

FIT (OC FIT-CHEK, Polymedco) was performed according to the manufacturer's instructions with the use of the same stool sample used for the DNA test.[20] Samples were refrigerated on receipt and sent in batches to a separate single laboratory for blinded analysis. Stool samples with more than 100 ng of hemoglobin per milliliter of buffer were considered to be positive.[20]

STATISTICAL ANALYSIS

The study was designed to have a power of 90% to test the prespecified hypothesis that the DNA test would have a sensitivity of 65% or more for the detection of colorectal cancer (AJCC stages I through IV) under the null hypothesis, at a one-sided type I error rate of 0.05. A secondary hypothesis was to rule out a 5% noninferiority margin for sensitivity for the detection of colorectal cancer with the DNA test as compared with FIT, at a one-sided type I error rate of 0.05. Testing of the two hypotheses with a power of at least 80% required the diagnosis of 49 and 56 adjudicated colorectal cancers, respectively, which required the enrollment of 10,500 to 12,000 participants, under the assumption of a colorectal-cancer prevalence of 4.5 cases per 1000 population.

We conducted prespecified analyses to determine the sensitivity of the multitarget DNA test, as compared with FIT, for the detection of screening-relevant colorectal cancer (AJCC stages I through III); the specificity of the multitarget

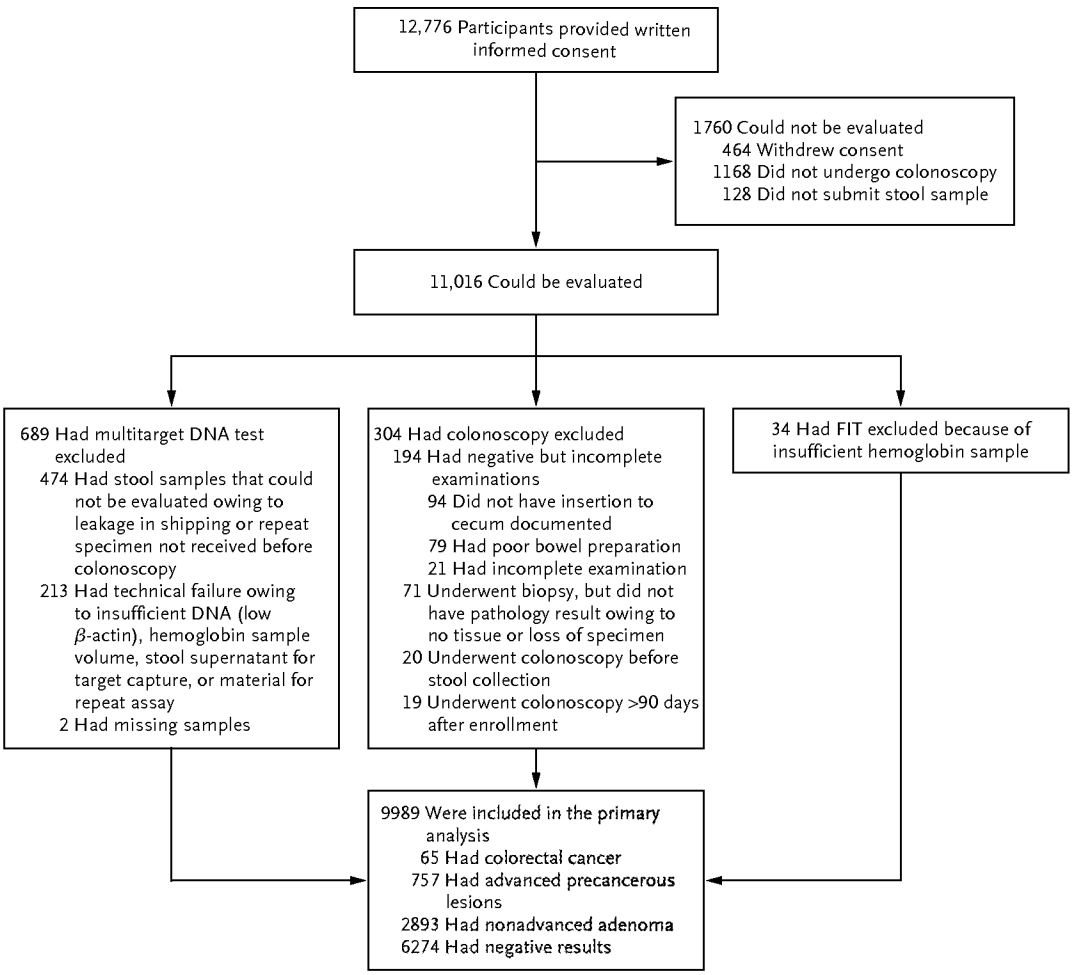

Figure 1. **Enrollment and Outcomes.**

DNA stool test (i.e., true negative rate), with advanced precancerous lesions on colonoscopy excluded and only nonadvanced adenomas and negative results included (the primary measure of specificity) and with only negative results included (the secondary measure of specificity); and the sensitivity of the multitarget stool DNA test, as compared with FIT, for the detection of advanced precancerous lesions. The analyses were based on data from all participants who had valid results on multitarget stool DNA testing, FIT, and colonoscopy; all reported subgroup analyses were prespecified.

For test characteristics, 95% lower boundaries were computed with the use of an exact binomial test. Lower 95% confidence limits for comparative analyses were computed with the use of a one-sided McNemar paired-comparisons test for the observed difference in sensitivity between the DNA test and FIT. The Hanley–McNeil method was used to calculate P values for the analysis of the receiver operating characteristic (ROC) curve.[21] There were no interim analyses of the data. All analyses were conducted with the use of SAS software, version 9.1, and StatXact software, version 7.

RESULTS

STUDY POPULATION

A total of 12,776 participants were enrolled at 90 sites; 9989 of these participants (78.2%) had results that could be fully evaluated (Fig. 1). The participants whose results could be fully evaluated and those whose results could not be fully evaluated differed significantly with respect to mean age and race, although the magnitudes of the differences were small (Table S1 in the Supplementary Appendix).

A total of 65 participants who could be evaluated were found to have colorectal cancer on colonoscopy (prevalence, 0.7%). Of these participants, 60 had screening-relevant (stage I to III) cancers. A total of 757 participants who could be evaluated had advanced precancerous lesions (prevalence, 7.6%).

DNA TEST CHARACTERISTICS

Multitarget stool DNA testing identified 60 of 65 participants with cancer, including 56 of the 60 participants with screening-relevant cancers, for respective sensitivities of 92.3% (95% confidence interval [CI], 83.0 to 97.5) and 93.3% (95% CI,

Table 1. **Sensitivity and Specificity of the Multitarget Stool DNA Test and the Fecal Immunochemical Test (FIT) for the Most Advanced Findings on Colonoscopy.**

Most Advanced Finding	Colonoscopy (N=9989)	Multitarget DNA Test (N=9989)		FIT (N=9989)	
		Positive Results	Sensitivity (95% CI)	Positive Results	Sensitivity (95% CI)
	no.	*no.*	%	*no.*	%
Colorectal cancer					
Any	65	60	92.3 (83.0–97.5)	48	73.8 (61.5–84.0)
Stage I to III*	60	56	93.3 (83.8–98.2)	44	73.3 (60.3–83.9)
Colorectal cancer and high-grade dysplasia	104	87	83.7 (75.1–90.2)	66	63.5 (53.5–72.7)
Advanced precancerous lesions†	757	321	42.4 (38.9–46.0)	180	23.8 (20.8–27.0)
Nonadvanced adenoma	2893	498	17.2 (15.9–18.6)	220	7.6 (6.7–8.6)
			Specificity (95% CI)		Specificity (95% CI)
All nonadvanced adenomas, non-neoplastic findings, and negative results on colonoscopy	9167	1231	86.6 (85.9–87.2)	472	94.9 (94.4–95.3)
Negative results on colonoscopy	4457	455	89.8 (88.9–90.7)	162	96.4 (95.8–96.9)

* These stages of colorectal cancer, as defined by the system recommended by the American Joint Committee on Cancer, are associated with an increased rate of cure.

† Advanced precancerous lesions include advanced adenomas and sessile serrated polyps measuring 1 cm or more.

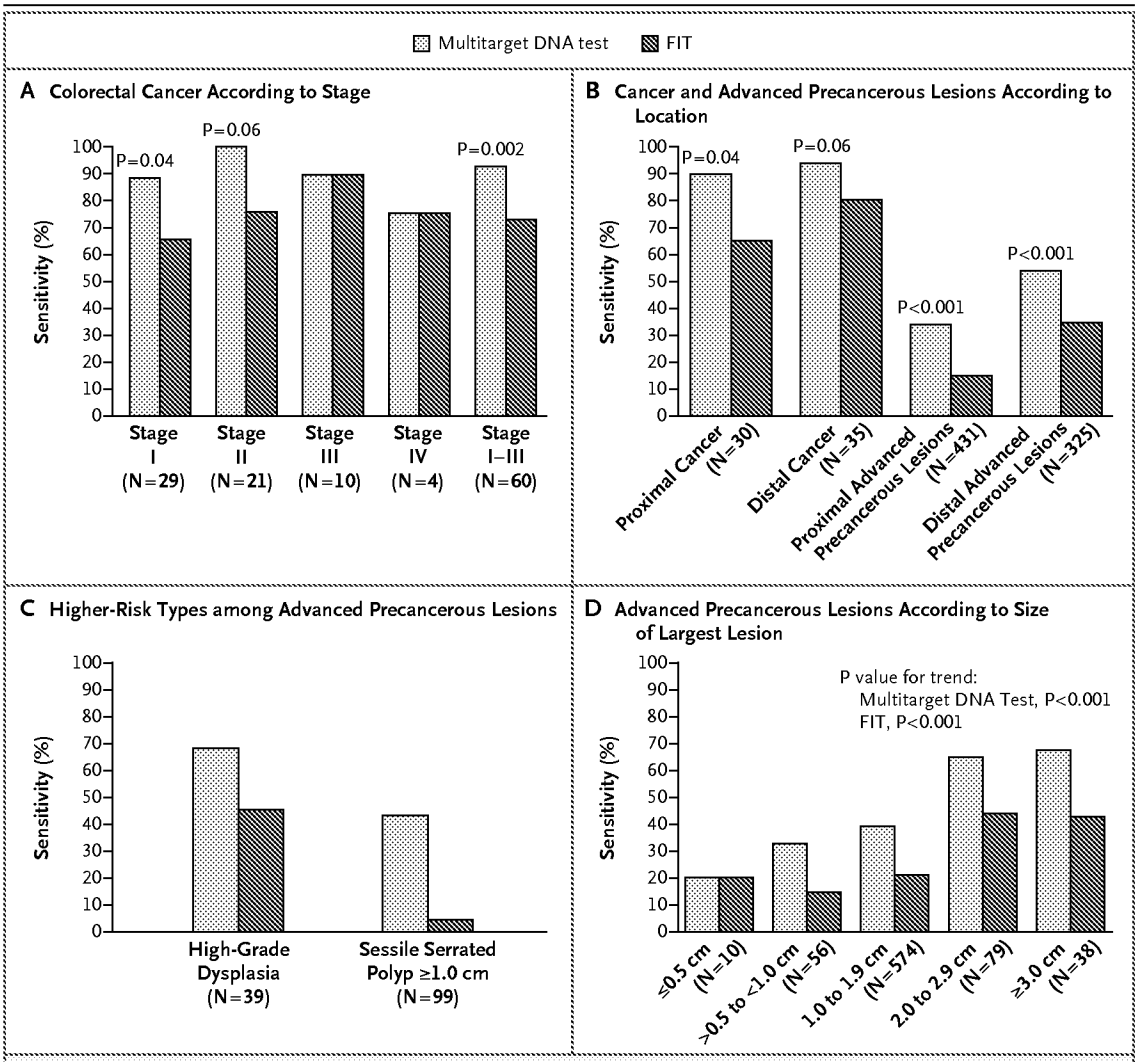

Figure 2. Sensitivity of the Multitarget Stool DNA Test and the Commercial Fecal Immunochemical Test (FIT), According to Subgroup.

Shown are the sensitivities of the DNA test and FIT for the detection of colorectal cancer according to tumor stage (Panel A), for the detection of colorectal cancer and advanced precancerous lesions according to the location in the colon (Panel B), and for the detection of higher-risk subtypes among participants with advanced precancerous lesions (Panel C) and according to lesion size (Panel D). The numbers in parentheses are the number of participants in each category. In Panel A, the stage of 1 of 65 colorectal cancers was not available. In Panel B, the location of 1 of 757 advanced precancerous lesions was not available.

83.8 to 98.2) (Table 1). Sensitivity did not vary significantly according to cancer stage (Fig. 2A) or location within the colon (Fig. 2B). Among 757 participants with advanced precancerous lesions, DNA testing detected 321 (42.4%; 95% CI, 38.9 to 46.0). A total of 69.2% (95% CI, 52.4 to 83.0) of 39 participants with high-grade dysplasia and 42.4% (95% CI, 32.6 to 52.8) of 99 participants with sessile serrated polyps measuring 1 cm or larger were identified on DNA testing (Fig. 2C). The sensitivity of the DNA test was higher for distal advanced precancerous lesions (177 of 325 [54.5%; 95% CI, 48.9 to 60.0]) than for proximal lesions (143 of 431 [33.2%; 95% CI, 28.8 to 37.8]) (Fig. 2B); test sensitivity increased as the lesion size increased (Fig. 2D). The sensitivity for the detection of cancer or advanced precancerous lesions did not differ significantly ac-

cording to age or laboratory-testing site (data not shown).

Among 9167 participants who had findings other than colorectal cancer or advanced precancerous lesions (e.g., nonadvanced adenomas or negative results), the specificity of the DNA test (true negative rate) was 86.6% (95% CI, 85.9 to 87.2). Among the 4457 participants with totally negative results on colonoscopy, the specificity was 89.8% (95% CI, 88.9 to 90.7); within this subgroup, the specificity was 94.0% among participants younger than 65 years of age and 87.1% among those 65 years of age or older (P<0.001).

COMPARISON WITH FIT

FIT detected 48 of 65 cancers (73.8%; 95% CI, 61.5 to 84.0), 44 of 60 AJCC stage I to III cancers (73.3%; 95% CI, 60.3 to 83.9), and 180 of 757 advanced precancerous lesions (23.8%; 95% CI, 20.8 to 27.0), findings that were all significantly inferior to those with DNA testing (Table 1). FIT detected 20 of 30 proximal cancers (66.7%) and 28 of 35 distal cancers (80.0%) (P=0.35 for the comparison between proximal and distal location). Comparative results for the detection of cancer according to stage and for higher-risk subsets of advanced precancerous lesions are shown in Figure 2. The DNA test was more sensitive than FIT for the detection of lesions with high-grade dysplasia (69.2% vs. 46.2%, P=0.004) or sessile serrated polyps measuring 1 cm or more (42.4% vs. 5.1%, P<0.001) (Fig. 2C) and for the detection of advanced precancerous lesions within the size ranges observed (Fig. 2D).

DNA testing detected 13 of 60 screening-relevant cancers that were undetected by FIT, whereas FIT detected 1 cancer that was undetected by DNA testing (P<0.001). DNA testing detected 170 of 757 advanced precancerous lesions (22.5%) that were undetected by FIT, whereas FIT detected 29 such lesions (3.8%) undetected by DNA testing (P<0.001).

Among 9167 participants with findings other than colorectal cancer or advanced precancerous lesions, the specificity of FIT was 94.9% (95% CI, 94.4 to 95.3). Among 4457 participants with negative results on colonoscopy, the specificity was 96.4% (95% CI, 95.8 to 96.9). In these two subgroups, the specificity values were superior to those of the DNA test (Table 1).

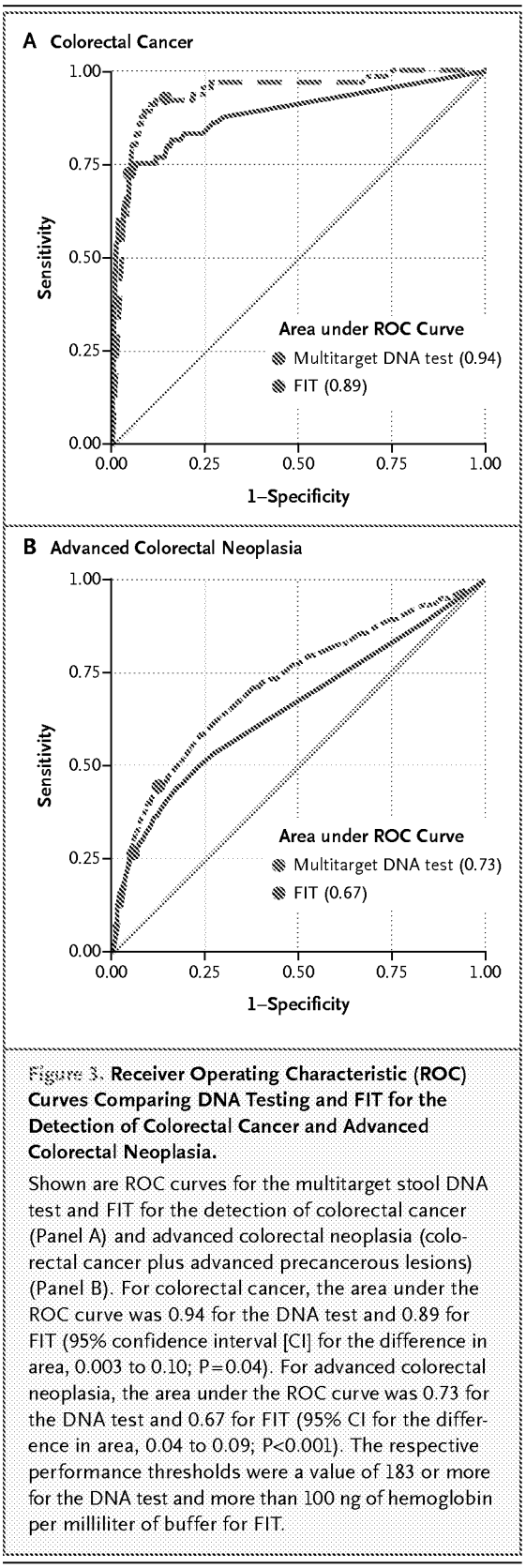

Figure 3. Receiver Operating Characteristic (ROC) Curves Comparing DNA Testing and FIT for the Detection of Colorectal Cancer and Advanced Colorectal Neoplasia.

Shown are ROC curves for the multitarget stool DNA test and FIT for the detection of colorectal cancer (Panel A) and advanced colorectal neoplasia (colorectal cancer plus advanced precancerous lesions) (Panel B). For colorectal cancer, the area under the ROC curve was 0.94 for the DNA test and 0.89 for FIT (95% confidence interval [CI] for the difference in area, 0.003 to 0.10; P=0.04). For advanced colorectal neoplasia, the area under the ROC curve was 0.73 for the DNA test and 0.67 for FIT (95% CI for the difference in area, 0.04 to 0.09; P<0.001). The respective performance thresholds were a value of 183 or more for the DNA test and more than 100 ng of hemoglobin per milliliter of buffer for FIT.

The specificity of FIT varied minimally according to age.

As measured by the area under the ROC curve (AUC), the discrimination between colorectal cancer and the combination of nonadvanced neoplasia and lesser findings was significantly higher with DNA testing than with FIT (0.94 vs. 0.89, P=0.04) (Fig. 3A); the AUC values for discrimination between advanced colorectal neoplasia (colorectal cancer plus advanced precancerous lesions) and all other findings were 0.73 and 0.67, respectively (P<0.001) (Fig. 3B). Positive and negative predictive values are shown in Table S2 in the Supplementary Appendix.

The isolated performance of the hemoglobin immunoassay component of the multitarget DNA test was similar to that of FIT, with specificities of 94.8% and 94.9%, respectively; sensitivities were 72.3% and 73.8%, respectively, for the detection of colorectal cancer and 22.7% and 23.8%, respectively, for the detection of advanced precancerous lesions.

Table 2 shows the number of persons who would need to be screened with colonoscopy, multitarget DNA testing, and FIT in order to detect one colorectal cancer (154 with colonoscopy, 166 with multitarget DNA testing, and 208 with FIT) and to detect one advanced precancerous polyp (13, 31, and 55 persons, respectively). These calculations show that multitarget DNA testing detected clinically significant lesions more efficiently than FIT.

EXTRAPOLATION TO AN EXPANDED SCREENING POPULATION

In an extrapolation of our results to a hypothetical reference population of 10,000 participants at average risk for colorectal cancer, the various screening techniques of colonoscopy, DNA testing, and FIT would identify, respectively, 65, 60, and 48 persons with colorectal cancer; 758, 321, and 180 persons with advanced precancerous lesions; 2896, 498, and 220 persons with nonadvanced adenomas; and 6281, 732, and 248 persons with non-neoplastic findings or negative results on colonoscopy (Table 3).

The protocol specified the detection of colorectal cancer and advanced precancerous polyps as positive findings and the detection of nonadvanced adenomas as negative findings. In the hypothetical reference population of 10,000 persons, the numbers of persons who would be referred for colonoscopy on the basis of positive test results would be 1611 (16.1%) with DNA testing and 696 (7.0%) with FIT. Of the positive test results, the numbers that would be viewed as false positives would be 1230 of 1611 (76.4%) with DNA testing and 468 of 696 (67.2%) with FIT. Of 8389 negative results for DNA testing, 442 (5.3%) would be viewed as false negatives, consisting of 5 cancers and 437 advanced precancerous polyps. Of 9304 negative results for FIT, 595 (6.4%) would be viewed as false negatives, consisting of 17 cancers and 578 precancerous polyps. If nonadvanced adenomas were considered to be positive findings, then the proportions of positive tests viewed as false positives would be 732 of 1611 (45.4%) with DNA testing and 248 of 696 (35.6%) with FIT. The numbers of negative tests viewed as false negatives would be 2840 of 8389 (33.9%) with DNA testing and 3271 of 9304 (35.2%) with FIT. Most of these false negative results would be small, nonadvanced adenomas (in 2398 of 2840 participants [84.4%] with DNA testing and 2676 of 3271 participants [81.8%] with FIT), with only rare instances of colorectal cancers (5 of 2840 [0.2%] and 17 of 3271 [0.5%], respectively).

DISCUSSION

We compared a multitarget stool DNA test with a commercial FIT among patients at average risk for colorectal cancer. The sensitivity of the DNA

Table 2. Numbers of Persons Who Would Need to Be Screened with Colonoscopy, Multitarget DNA Test, and FIT to Detect One Colorectal Cancer and One Advanced Precancerous Lesion.

Finding	Number Needed to Screen (95% CI)		
	Colonoscopy	Multitarget DNA Test	FIT
Any colorectal cancer	154 (120–200)	166 (130–217)	208 (156–286)
Stage I to III colorectal cancer	166 (130–217)	178 (140–238)	227 (169–313)
Advanced precancerous lesion	13 (12–14)	31 (28–35)	55 (48–65)

Table 3. **Extrapolation of Findings to an Expanded Population of 10,000 Persons at Average Risk for Colorectal Cancer Undergoing Screening with Colonoscopy, Multitarget Stool DNA Test, and FIT.***

Colonoscopy Finding	Persons with Finding	Multitarget DNA Test		FIT	
		Positive Results (N=1611)	Negative Results (N=8389)	Positive Results (N=696)	Negative Results (N=9304)
	no.	*no. (%)*			
Colorectal cancer	65	60 (3.7)	5 (0.06)	48 (6.9)	17 (0.18)
Advanced precancerous lesions	758	321 (19.9)	437 (5.2)	180 (25.9)	578 (6.2)
Nonadvanced adenomas	2896	498 (30.9)	2398 (28.6)	220 (31.6)	2676 (28.8)
Negative results: no colorectal cancer, advanced precancerous lesions, or nonadvanced adenomas	6281	732 (45.4)	5549 (66.1)	248 (35.6)	6033 (64.8)

* Listed are data from the study that have been extrapolated to a theoretical population of 10,000 persons.

test for the detection of both colorectal cancer (92.3%) and advanced precancerous lesions (42.4%) exceeded that of FIT by an absolute difference of nearly 20 percentage points. This difference may be attributed to the DNA marker and algorithm components of the test, since the test performance of the hemoglobin immunoassay component of the DNA test was nearly identical to that of FIT. However, FIT was more specific for the detection of both colorectal cancer and advanced precancerous lesions, by absolute differences of 6.6 to 8.3 percentage points.

Sensitivity is the most important characteristic for screening tests because the primary role of such testing is to rule out diseases such as cancer. The sensitivity of the DNA test for the detection of advanced precancerous lesions was approximately half that for the detection of colorectal cancer; it exceeded the performance of the FIT overall and in important subsets of lesions, including adenomas measuring 2 cm or more (in which the prevalence of high-grade dysplasia is 5 to 44%[22-25]) and large, sessile serrated polyps (which may account for up to one third of colorectal cancers[26,27]). In our study, DNA testing was associated with a relative increase of 27% in the rate of detection of stage I to III colorectal cancers and a relative increase of 78% in the rate of detection of advanced precancerous lesions, as compared with FIT. A negative result on DNA testing reduced the chance of having colorectal cancer to a greater extent than did a negative result on FIT, from a baseline risk of approximately 1 in 154 (0.7%) to 1 in 1675 (0.06%) after DNA testing and 1 in 556 (0.18%) after FIT.

Although high sensitivity is the most important attribute of cancer-screening tests, specificity is also important, since it affects the number of persons who have positive test results, a majority of whom will have false positive results because of the low prevalence of cancer. The specificity of FIT (94.9 to 96.4%) was superior to that of the DNA test (86.6 to 89.8%), with false positive rates of 3.6 to 5.1% and 10.2 to 13.4%, respectively. Positive results on the DNA test increased the probability of having colorectal cancer from 0.7% to 3.7%, as compared with 6.9% for FIT, and increased the probability of having an advanced precancerous lesion from 7.3% to 19.9%, as compared with 25.9% for FIT.

Two points regarding the specificity of DNA testing deserve comment. First, analysis of the primary measure of specificity (86.6%) included participants with nonadvanced adenomas, which can cause the test to be positive, and those with a negative result on colonoscopy. Among persons with only a negative result on colonoscopy, the specificity of the DNA test was nearly 90%, although that was still inferior to the specificity of FIT, which exceeded 96%. Second, specificity correlated inversely with age. Among participants with any findings other than advanced neoplasia, specificity varied from 91.5% among participants between the ages of 50 and 64 years to 83.7% among those 65 years of age or older. Among persons 50 to 64 years of age with a negative result on colonoscopy, the specificity of

the DNA test was 94%, which was similar to that of FIT. Age-related variation in specificity could be due to the presence of lesions that were missed on colonoscopy (which are more prevalent among persons older than 70 years[28]) or to age-related DNA methylation.[29,30]

The performance of FIT in our study was similar to that in previous studies in which colonoscopy was the reference standard.[31,32] For FIT in our study, we used the manufacturer's threshold of 100 ng of hemoglobin per milliliter of buffer.[20] Whether results would differ with the use of another type of FIT is not known; however, the detection rate with the FIT we used was higher than the rate with at least one other FIT.[33]

A discussion of the role of multitarget stool DNA testing in colorectal-cancer screening is beyond the scope of this report because it requires the assessment of several factors aside from sensitivity and specificity, which are the focus of this report. Other factors include performance characteristics of alternative tests, testing intervals, complications, costs, patient acceptance, and adherence.[34,35] Downstream effects of these factors on outcomes, including both cause-specific and overall morbidity and mortality, require modeling studies to compare various screening tests and strategies. Although our study provides some of the important values for modeling, it cannot determine which test or strategy is better or preferred.

Screening rates for colorectal cancer remain low despite strong evidence of the effectiveness of several tests and strategies. The U.S. Preventive Services Task Force states that there is no preferable screening test, as supported by several cost-effectiveness analyses.[5-7] Offering a choice among tests may improve uptake of screening.[32,36] A noninvasive test with a high single-application sensitivity for curable-stage cancer may provide an option for persons who prefer noninvasive testing. Questions about testing intervals and tailoring require further consideration.

In conclusion, a stool test combining altered human DNA and fecal hemoglobin showed higher single-application sensitivity than a commercial FIT for both colorectal cancer and advanced precancerous lesions, although with lower specificity.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Houston, TX; D. Whitebloom, Regional Gastroenterology Associates of Lancaster, Lancaster, PA; W. Bray, Digestive Health Specialists, Winston-Salem, NC; H. Schwartz, Miami Research Associates, Miami, FL; M. LeVine, Atlanta Gastroenterology Associates, Marietta, GA; R. Bennetts, Northwest Gastroenterology Clinic, Portland, OR; T. Kun, Santa Monica Research Institute, Santa Monica, CA; J. Stern, Commonwealth Clinical Studies, Brockton, MA; V. Kodali, Cumberland Research Associates, Fayetteville, NC; Z. Younes, Gastro One, Germantown, TN; S. Ojha, Southern California Oncology, Downey, CA; T.C. Trate, MediSpect, Boone, NC; C. Lang, NCH Medical Group, Arlington Heights, IL; J. Kittinger, Wilmington Gastroenterology Associates, Wilmington, NC; T. Schnell, Hines VA Hospital, Hines, IL; R. Barnett, Advanced Research Institute, Sandy, UT; C. Rall, Marshfield Clinic, Marshfield, WI; L. Bank, Binghamton Gastroenterology, Binghamton, NY; K. Lee; REMEK Research, Pomona, CA; C. Strout, Coastal Carolina Research Center, Mount Pleasant, SC; J. Ziebert, Professional Quality Research, Austin, TX; M. Jardula; Desert Oasis Healthcare, Palm Springs, CA; R. Wohlman, Northwest Gastroenterology Associates, Bellevue, WA; J. Lowe, Advanced Research Institute, South Ogden, UT; W. Essilfie, Healthcare Partners Medical Group, Torrance, CA; H. Gagneja, Professional Quality Research, Austin, TX; R. Be, Gastroenterology Associates, Baton Rouge, LA; S. Itzkowitz, Mount Sinai School of Medicine, New York, NY; V. Koduri, Clinical Research Associates of Tidewater, Norfolk, VA; B. Kaufman, AGA Clinical Research Associates, Egg Harbor Township, NJ; S. Geller, Centennial Medical Group, Elkridge, MD; M. Mastrangelo, Hanover Medical Specialists, Wilmington, NC; R. Iyer, Iowa Digestive Disease Center, Clive, IA; O. Zaidel, South Bay Gastroenterology Medical Group, Torrance, CA; P. Nandi, Center for Digestive Health, Troy, MI; C. Gross, Desert Sun Gastroenterology, Tucson, AZ; R. Heigh, Mayo Clinic Scottsdale, Scottsdale, AZ; X. Llor, University of Illinois at Chicago/Jesse Brown VA, Chicago, IL; A. Focil, Diverse Research Solutions, Oxnard, CA; D. Riegert-Johnson, Mayo Clinic Jacksonville, Jacksonville, FL; M. Goldstein, Gastrointestinal Research Group, Great Neck, NY; H. Michael, Summit Medical Group, Berkeley Heights, NJ; J. Wolosin; Sharp-Rees-

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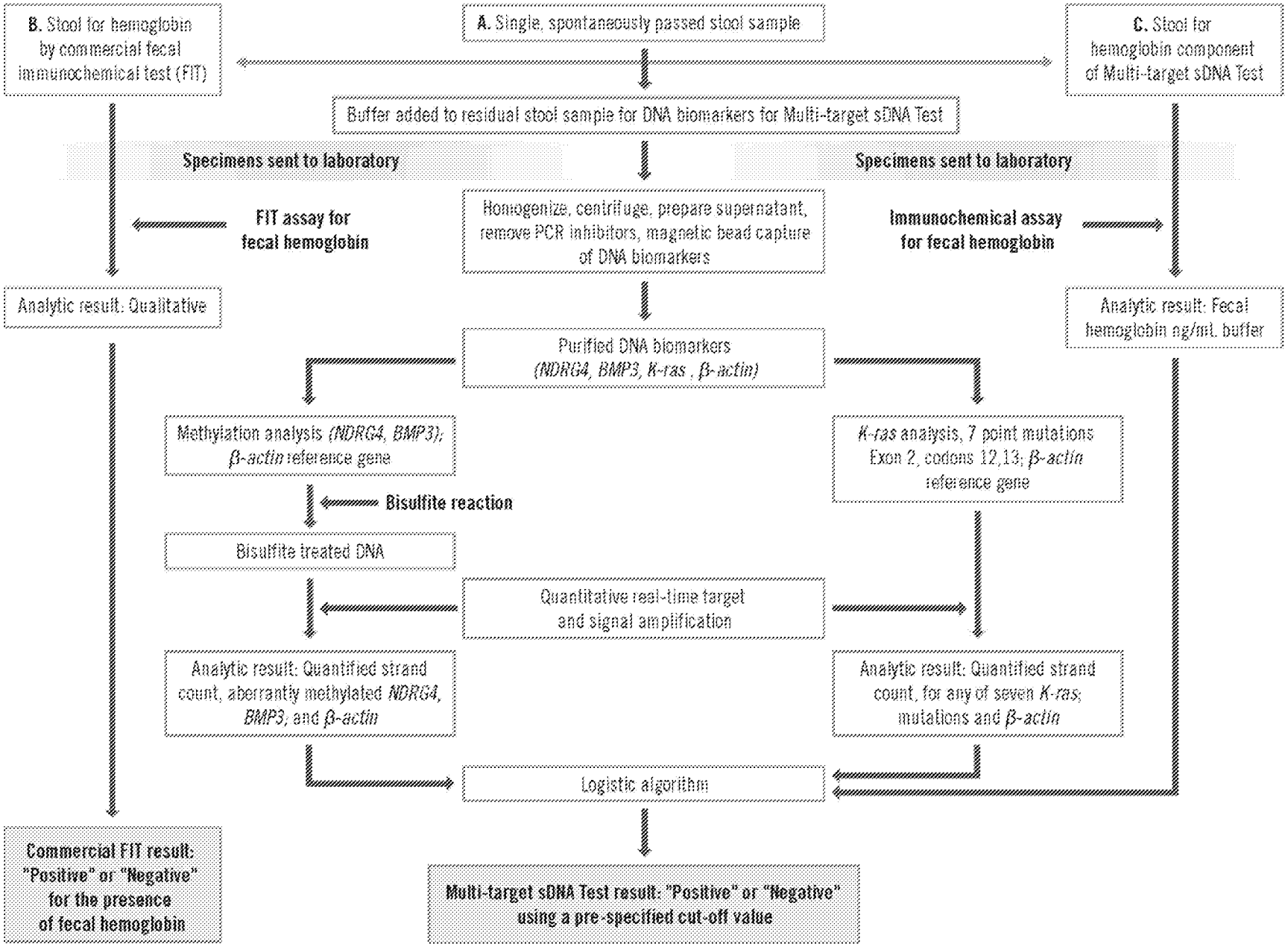

Supplemental Figure Legend

Figure S1. Study Specimen Flow and Approach to Extraction and Analysis of Stool DNA and Hemoglobin. Study participants passed a single spontaneous stool sample into the provided collection container (A.), sampled the stool for the commercial FIT test (B.) and for the hemoglobin component of the multi-target stool DNA test (C.) and poured a DNA stabilization buffer onto the remaining stool sample. All three samples were returned to the laboratory together for blinded analyses. The commercial FIT test was performed per manufacturer's instructions which provides a qualitative result of positive or negative for the presence of fecal hemoglobin based on a quantitative result with a cut-off value of 100 ng Hb / ml buffer. The hemoglobin component of the multi-target sDNA test was analyzed by a separate immunochemical assay. The buffered residual stool sample was normalized with additional buffer to a 1 gm:5 ml stool: buffer ratio and homogenized. Homogenates were aliquoted 42 ml/tube and frozen at -80^{o}C until analyzed. Aliquots were thawed at room temperature, centrifuged and the supernatant was treated with polyvinylpyrrolidone to remove PCR inhibitors and spin filtered to clarify. Sequence specific DNA biomarker targets were isolated directly from the clarified supernatant using a magnetic bead based oligonucleotide hybrid capture method. The captured DNA was split and one portion was subjected to the bisulfite reaction for the identification of aberrantly methylated *NDRG4* and *BMP3* and the other portion was used to determine the presence of any of seven *K-ras* point mutations. The test does not distinguish the specific mutation. Using quantitative real-time target and signal amplification, *β-Actin* for total DNA content and methylated *NDRG4* and *BMP3* and were analyzed in one well of a 96 well PCR plate while β-*Actin* for total DNA content and 7 *K-ras* point mutations were analyzed in a second well of the same plate. The results of this analysis for methylated *NDRG4*, methylated *BMP3*, mutated *K-ras* and *β-Actin* in log strands was combined with the results of the stool DNA test hemoglobin component in ng/ml of buffer in a pre-specified logistic regression algorithm, the output of which determined if the stool DNA test was positive, if the pre-specified cut-off value was ≥ 183, or negative..

Table S1. Demographic characteristics of enrolled population

Characteristic	Evaluable group (n=9,989)	Non-Evaluable group (n=2753)
Age (mean [SD])	64.2 [8.41]	65.4 [8.50]
50-59 yr – no. (%)	2862 (28.7)	700 (25.4)
60-64 yr – no. (%)	819 (8.2)	196 (7.1)
65-69 yr – no. (%)	3670 (36.7)	986 (35.8)
70-74 yr – no. (%)	1735 (17.4)	497 (18.1)
≥ 75 yr – no. (%)	903 (9.0)	374 (13.6)
Male sex – no. (%)	4625 (46.3)	1282 (46.6)
Race – no. (%)		
Caucasian	8392 (84.0)	2080 (75.6)
African-American	1068 (10.7)	480 (17.5)
Other	523 (5.2)	190 (6.9)

Table 2. Positive and negative predictive values of multi-target stool DNA and fecal immunochemical test for colorectal cancer and advanced colorectal neoplasia*

		Positive Predictive Value (95% CI)		Negative Predictive Value (95% CI)	
Outcome	n of lesions / N of subjects	Multi-target stool DNA	Commercial FIT	Multi-target stool DNA	Commercial FIT
Colorectal cancer	65 / 9989	0.037 (0.029-0.048)	0.069 (0.051-0.090)	0.999 (0.998-1.00)	0.998 (0.997-0.999)
Advanced colorectal neoplasia*	822 / 9989	0.236 (0.216-0.258)	0.326 (0.291-0.362)	0.947 (0.942-0.952)	0.936 (0.931-0.941)

Methodology for calculating the multi-target stool DNA test composite score: The multi-target stool DNA algorithm

Overview:

The clinical performance of the multi-target stool DNA test reported in this study derives from the combination of the technical sensitivity and specificity of the analytic methods, which provide the patient's DNA and fecal hemoglobin biomarker values, and the integration of those values by a mathematical algorithm, which calculates the final result, the composite score The analytic methods and algorithm development are described in detail in the supplemental materials and in previous publications. [1, 2] The series of calculations, constants and conditions described below (collectively referred to as the multi-target stool DNA composite score algorithm (the Algorithm) have been incorporated into the multi-target stool DNA analytic device software. While this algorithmic approach to data analysis is a general one, the specific constants and thresholds are specific for the analytic methods used for the multi-target stool DNA test, as reported in this study. If the Algorithm were applied to data from analytic methods with a different technical sensitivity and specificity than those used in the current study, the constants and thresholds would need to be re-determined on a training set of stool specimens including those from patients with colorectal cancer, pre-malignant lesions and normal colonoscopies to achieve a similar clinical discrimination between patients with colorectal and advanced pre-cancerous lesions and those without those lesions.

The Algorithm and all supporting analytic methods were presented to the U.S. Food and Drug Administration on February 17, 2012 as part of the FDA pre-market approval application process for the multi-target stool DNA test. The Algorithm and weighting factors were locked into the software February 22, 2013. Study sample testing was initiated in April 2013. All results presented in the study were calculated based on this Algorithm, which is provided below.

The Algorithm has three components. The heart of the algorithm and first component is a logistic regression formula which allows the biomarkers, as a group, to be used to discriminate between patients with colorectal cancer and/or advanced precancerous lesions (defined in the text of the manuscript) from negative cases, which include all other findings and negative colonoscopic examinations. The result of this calculation is the Logistic Score.

The second component calculates a Sum of Scores that incorporates the Logistic Score and individual marker scores for the DNA markers. These DNA biomarker scores are entered as either "0" or "10" depending on whether the DNA marker is below ("0") or above ("10") the 99.5 percentile of normal. The Sum of Scores calculation ensures that if any one DNA marker is above the 99.5 percentile of normal (boundary condition), that the test will have a "positive" result.

Finally, in the third component, the Sum of Scores is subjected to an exponential equation which generates the final Composite Score. This creates a range of values from 0 – 1000 for the multi-target sDNA test Composite Score (reference range: 0-182 for a "negative" result). If the Composite Score is ≥ 183, the multi-target stool DNA result is "positive". If the Score is < 183, the result is "negative". The multi-target stool DNA test only provides a dichotomous "positive" or "negative" result. No other information is provided.

Calculations:

Seven analytical parameters are used. The multi-target stool DNA assay consists of 2 triplex QuARTS assays for DNA biomarkers (quantitative allele specific real-time target and signal amplification) [2] and an immunochemical assay for fecal hemoglobin. The first QuARTS assay quantifies the number of DNA strands of aberrantly methylated *NDRG4* and *BMP3* gene promoter region and quantifies the number of strands of *β-actin* gene, which acts *as a* reference gene and a measure of total human DNA (referred to as ACTB ANB in the formulas). The second QuARTS assay quantifies the strands of DNA containing one or more of seven *K-ras* point mutations in Exon 2, codons 12 and 13, divided into two groups, a group of three mutations and a group of four mutations. The *Kras* assay does not determine which specific *K-ras* mutation(s) is present. This

QuARTS assay also includes *β-actin* quantification (called ACTB *K-ras* in the formula).

The *β-actin* strand counts are used as a control to determine if sufficient DNA was isolated and analyzed in each QuARTS assay and as a measure of total human DNA. If the *β-actin* strand count in either QuARTS assay is not ≥ 2.301 log strands then the sample is considered invalid and no score is calculable. As a measure of human DNA content, *β-actin* strand count is also a parameter in the logistic regression calculation. The strand count from only one QuARTS assay is needed and for consistency the *β-actin* from the QuARTS methylation assay (ACTB ANB) is used.

The calculation process for the multi-target stool DNA composite Score is illustrated in Figure 1. Sample data from study cases, the resulting calculated Composite Scores from the Algorithm and the corresponding multi-target stool DNA test result, and the study colonoscopic findings are listed in Table S3.

Using the Algorithm to calculate the multi-target stool DNA Composite Score

Step 1. Record the results of the QuARTS methylation and Kras mutation assays and the fecal hemoglobin assay

Step 2. Determine if sufficient β-actin (ACTB ANB and ACTB Kras) is present in each QuARTS assay for valid test results,

Step 3. Calculate the Logistic Score as per the formula

Step 4. Determine the BMP3, NDRG4, KRAS1 and KRAS 2 individual scores (either 0 or 10)

Step 5. Calculate the Sum of Scores

Step 6 Calculate the Composite Score

Step 7. Assign the specimen a result of positive (Composite Score ≥ 183) or negative (Composite Score < 183).

References:

1. Lidgard GP, Domanico MJ, Bruinsma JJ, et al. Clinical Performance of an Automated Stool DNA Assay for Detection of Colorectal Neoplasia. Clin Gastroenterol Hepatol 2013.
2. Zou H, Allawi H, Cao X, Quantification of methylated markers with a multiplex methylation specific technology. Clin Chem 2012; 58:375 -383.
3. *See* Mansfield, E. "Novel Diagnostics: FDA Perspectives," p.13, http://www.cmod.org/assets/2007_Secure_Files/CV2007Mansfield.pdf (accessed on 8/03/12).

Figure S2. Multi-target sDNA Algorithm – Composite Score Calculation

		Multi-target stool DNA QuARTS assay for methylation markers			Multi-target stool DNA QuARTS assay for Kras mutation marker			Multi-target stool DNA assay for fecal hemoglobin
Logistic Score inputs and calculations*	**Biomarker Strand Counts or fecal hemoglobin in ng/ml buffer****	BMP3 strands	NDRG4 strands	β-actin (ACTB ANB) strands	Kras 1 strands	Kras 2 strands	β-actin (ACTB Kras) strands	Fecal hemoglobin ng/ml
	Weighting factors	X1 = 0.990944982	X2 = 0.790758688	X5 = -0.392492543	X3 = 1.119602381	X4 = 0.428424885		X6 = 0.008894634
	Logistic Score formula	*X1*Log10(BMP3 strands+1)+X2*Log10(NDRG4 strands+1)+X3*Log10(KRAS1 strands +1)+X4*Log10(KRAS2 strands+1)+X5*Log10(100/(ACTB ANB strands +1))+X6*Hemoglobin ng/ml - 2.796044521*						
Sum of Scores inputs and calculations	**DNA marker scores in the Sum of Score Formula are either 0 or 10:** BMP3_Score, NDRG4_Score, Kras_1 Score, Kras_2 score	If :Log10 BMP3 strands ≥ Log10(0.029294806) + Log10 ACTB ANB strands, 10, else 0	If :Log10 NDRG4 strands ≥ Log10(0.112083742) + Log10 ACTB ANB strands, 10, else 0		If :Log10 KRAS1 strands ≥ Log10(0.043660902) + Log10 ACTB KRAS strands, 10, else 0	If :Log10 KRAS2 strands ≥ Log10(0.074733554) + Log10 ACTB KRAS strands, 10, else 0		
	Sum of Scores formula	Sum of Scores = Logistic Score + BMP3_Score + NDRG4_Score + KRAS1_Score + KRAS2_Score						
Composite Score inputs and calculations	**Composite Score formula**	$(e^{\text{Sum of Scores}}) / (1+e^{\text{Sum of Scores}})) * 1000$ = multi-target stool DNA Composite Score						
Multi-target stool DNA result determination	**Reference range: Negative <183**	**If the Composite Score <183, result as "Negative; If Composite Score ≥ 183 result as "Positive"**						

* Quality Measures:

1. If β-actin (ACTB ANB or ACTB KRAS) < 2.301 log strands, the sample is invalid as there is insufficient human DNA for accurate analysis
2. If Log10 ACTB KRAS -Log10 ACTB ANB <-0.52 or > 1.04, the sample is invalid as there has been a recovery of error for β-actin (ACTB)

** The dynamic range of the DNA assays is 10-300,000 strands. If the DNA marker value is below the dynamic range (<10 strands), the biomarker value is set to 0 for the Logistic Score calculation. If the DNA marker value is >300,000 strands, the biomarker value is set to 300,000 for the Logistic Score calculation. The dynamic range of the fecal hemoglobin assay is 6ng/ml - 500 ngml. If the fecal hemoglobin level is below 6 ngml, the value is set to 0 ng/ml and if the hemoglobin vlaue is >500 ng/ml, the hemiglobin value is set to 500 for calculating the Logistic Score.

Table S3. Sample multi-target stool DNA biomarker analytic data, Composite Score and Clinical Findings

	Multi-target stool DNA analytic data									
	Multiplex QuARTS Reaction: Methylation markers			Multiplex QuARTS Reaction: Kras mutation markers			Immunochemical fecal hemoglobin assay			
DeeP-C study sample case	BMP3 strand count	NDRG4 strand count	β-actin (ACT ANB) strand count	KRAS1 strand count	KRAS2 strand count	β-actin (Kras ACT) strand count	mullti-target sDNA fecal hemolgobin ng/ml buffer	Mullti-target stool DNA Composite Score	Mullti-target stool DNA result	DeeP-C Study Colonoscopic Finding
Case 1	49	695	7704	59	113	12239	3	264	Positive	Colorectal cancer
Case 2	64	166	2614	4	3	4070	18	339	Positive	Colorectal cancer
Case 3	77	472	2908	62	474	6629	10	1000	Positive	Adenoma with high-grade dysplasia
Case 4	45	125	1138	0	0	4820	0	1000	Positive	Sessile serrated polyp ≥ 1.0 cm
Case 5	0	2	226	0	0	907	77	75	Negative	Tubular adenoma < 1.0 cm
Case 6	3	42	10279	29	61	18634	24	26	Negative	Negative

U.S. Food and Drug Administration
Protecting and Promoting *Your* Health

FDA News Release

FDA approves first non-invasive DNA screening test for colorectal cancer

Collaboration with CMS contributed to proposed Medicare coverage

For Immediate Release

August 11, 2014

Release

Español (/NewsEvents/Newsroom/ComunicadosdePrensa/ucm409170.htm)

The U.S. Food and Drug Administration today approved Cologuard, the first stool-based colorectal screening test that detects the presence of red blood cells and DNA mutations that may indicate the presence of certain kinds of abnormal growths that may be cancers such as colon cancer or precursors to cancer.

Colorectal cancer primarily affects people age 50 and older, and among cancers that affect both men and women, it is the third most common cancer and the second leading cause of cancer-related death in the United States, according to the Centers for Disease Control and Prevention (CDC). Colorectal cancer screening is effective at reducing illness and death related to colon cancer. The CDC estimates that if everyone age 50 or older had regular **screening tests (http://www.cdc.gov/cancer/colorectal/basic_info/screening/tests.htm)** as recommended, at least 60 percent of colorectal cancer deaths could be avoided.

Colorectal cancer occurs in the colon (large intestine) or rectum (the passageway that connects the colon to the anus). Most colorectal cancers start as abnormal raised or flat tissue growths on the wall of the large intestine or rectum (polyps). Some very large polyps are called advanced adenomas and are more likely than smaller polyps to progress to cancer.

Using a stool sample, Cologuard detects hemoglobin, a protein molecule that is a component of blood. Cologuard also detects certain mutations associated with colorectal cancer in the DNA of cells shed by advanced adenomas as stool moves

through the large intestine and rectum. Patients with positive test results are advised to undergo a diagnostic colonoscopy.

“This approval offers patients and physicians another option to screen for colorectal cancer,” said Alberto Gutierrez, Ph.D., director of the Office of In Vitro Diagnostics and Radiological Health at the FDA’s Center for Devices and Radiological Health. “Fecal blood testing is a well-established screening tool and the clinical data showed that the test detected more cancers than a commonly used fecal occult test.”

Today’s approval of the Cologuard does not change current practice guidelines for colorectal cancer screening. Stool DNA testing (also called “fecal DNA testing”) is not currently recommended as a method to screen for colorectal cancer by the United States Preventive Services Task Force (USPSTF). Among other guidelines, the USPSTF recommends adults age 50 to 75, at average risk for colon cancer, be screened using fecal occult blood testing, sigmoidoscopy, or colonoscopy.

The safety and effectiveness of Cologuard was established in a clinical trial that screened 10,023 subjects. The trial compared the performance of Cologuard to the fecal immunochemical test (FIT), a commonly used non-invasive screening test that detects blood in the stool. Cologuard accurately detected cancers and advanced adenomas more often than the FIT test. Cologuard detected 92 percent of colorectal cancers and 42 percent of advanced adenomas in the study population, while the FIT screening test detected 74 percent of cancers and 24 percent of advanced adenomas. Cologuard was less accurate than FIT at correctly identifying subjects negative for colorectal cancer or advanced adenomas. Cologuard correctly gave a negative screening result for 87 percent of the study subjects, while FIT provided accurate negative screening results for 95 percent of the study population.

Today the Centers for Medicare & Medicaid Services (CMS) issued a proposed national coverage determination for Cologuard. Cologuard is the first product reviewed through a joint FDA-CMS pilot program known as parallel review where the agencies concurrently review medical devices to help reduce the time between the FDA’s approval of a device and Medicare coverage. This voluntary pilot program is open to certain premarket approval applications for devices with new technologies and to medical devices that fall within the scope of a Part A or Part B Medicare benefit category and have not been subject to a national coverage determination.

“Parallel review allows the last part of the FDA process to run at the same time as the CMS process, cutting as many as six months from the time from study initiation to coverage,” said Nancy Stade, CDRH’s deputy director for policy. “The pilot program is ongoing, but we will apply what we have learned to improve the efficiency of the medical device approval pathway for devices that address an important public health need.”

“This is the first time in history that FDA has approved a technology and CMS has proposed national coverage on the same day,” said Patrick Conway, chief medical officer and deputy administrator for innovation and quality for CMS. “This parallel review represents unprecedented collaboration between the two agencies and industry and most importantly will provide timely access for Medicare beneficiaries to an innovative screening test to help in the early detection of colorectal cancer.”

CMS proposes to cover the Cologuard test once every three years for Medicare beneficiaries who meet all of the following criteria:

- age 50 to 85 years,
- asymptomatic (no signs or symptoms of colorectal disease including but not limited to lower gastrointestinal pain, blood in stool, positive guaiac fecal occult blood test or fecal immunochemical test), and
- average risk of developing colorectal cancer (no personal history of adenomatous polyps, of colorectal cancer, or inflammatory bowel disease, including Crohn's Disease and ulcerative colitis; no family history of colorectal cancers or an adenomatous polyp, familial adenomatous polyposis, or hereditary nonpolyposis colorectal cancer).

Cologuard is manufactured by Exact Sciences in Madison, Wisconsin.

The FDA, an agency within the U.S. Department of Health and Human Services, protects the public health by assuring the safety, effectiveness, and security of human and veterinary drugs, vaccines and other biological products for human use, and medical devices. The agency also is responsible for the safety and security of our nationâ€™s food supply, cosmetics, dietary supplements, products that give off electronic radiation, and for regulating tobacco products.

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Inquiries

Media

Jenny Haliski (mailto:Jennifer.Haliski@fda.hhs.gov)
301-796-0776

Consumers

888-INFO-FDA

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Cologuard®

sDNA-based Colorectal Cancer Screening Test

Instructions for Use

IVD

For *in vitro* diagnostic use

Exact Sciences Corporation

441 Charmany Drive

Madison, WI 53719

www.exactsciences.com

844-870-8878

Table of Contents

Intended Use and Indications for Use

Intended Use

Cologuard is intended for the qualitative detection of colorectal neoplasia associated DNA markers and for the presence of occult hemoglobin in human stool. *Cologuard* is for use with the *Cologuard* collection kit and the following instruments: BioTek ELx808 Absorbance Microplate Reader; Applied Biosystems® 7500 Fast Dx Real-Time PCR; Hamilton Microlab® STARlet; and the Exact Sciences System Software with *Cologuard* Test Definition.

Indications for Use

Cologuard is intended for the qualitative detection of colorectal neoplasia associated DNA markers and for the presence of occult hemoglobin in human stool. A positive result may indicate the presence of colorectal cancer (CRC) or advanced adenoma (AA) and should be followed by diagnostic colonoscopy. *Cologuard* is indicated to screen adults of either sex, 50 years or older, who are at typical average-risk for CRC*Cologuard* is not a replacement for diagnostic colonoscopy or surveillance colonoscopy in high risk individuals.

Contraindications

Cologuard is intended for use with patients, age 50 years and older, at average risk who are typical candidates for CRC screening. *Cologuard* was not clinically evaluated for the following types of patients:

- Patients with a history of colorectal cancer, adenomas, or other related cancers.
- Patients who have had a positive result from another colorectal cancer screening method within the last 6 months.
- Patients who have been diagnosed with a condition that is associated with high risk for colorectal cancer. These include but are not limited to:
    - Inflammatory Bowel Disease (IBD)
    - Chronic ulcerative colitis (CUC)
    - Crohn's disease
    - Familial adenomatous polyposis (FAP)
    - Family history of colorectal cancer
- Patients who have been diagnosed with a relevant familial (hereditary) cancer syndrome, such as Hereditary non-polyposis colorectal cancer syndrome (HNPCCC or Lynch Syndrome), Peutz-Jeghers Syndrome, MYH-Associated Polyposis (MAP), Gardner's syndrome, Turcot's (or Crail's) syndrome, Cowden's syndrome, Juvenile Polyposis, Cronkhite-Canada syndrome, Neurofibromatosis, or Familial Hyperplastic Polyposis.

Warnings and Precautions

- The performance of *Cologuard* has been established in a cross sectional study (i.e., single point in time). Programmatic performance of *Cologuard* (i.e., benefits and risks with repeated testing over an established period of time) has not been studied. Performance has not been evaluated in adults who have been previously tested with

Cologuard. Non-inferiority or superiority of *Cologuard* programmatic sensitivity as compared to other recommended screening methods for CRC and AA has not been established.

- CRC screening guideline recommendations vary for persons over the age of 75. The decision to screen persons over the age of 75 should be made on an individualized basis in consultation with a healthcare provider. *Cologuard* test results should be interpreted with caution in older patients as the rate of false positive results increases with age.
- A negative *Cologuard* test result does not guarantee absence of cancer or advanced adenoma. Patients with a negative *Cologuard* test result should be advised to continue participating in a colorectal cancer screening program with another recommended screening method. The screening interval for this follow-up has not been established.
- *Cologuard* may produce false negative or false positive results. A false positive result occurs when *Cologuard* produces a positive result, even though a colonoscopy will not find cancer or precancerous polyps. A false negative result occurs when *Cologuard* does not detect a precancerous polyp or colorectal cancer even when a colonoscopy identifies the positive result.
- Patients should not provide a sample for *Cologuard* if they have diarrhea or if they have blood in their urine or stool (e.g., from bleeding hemorrhoids, bleeding cuts or wounds on their hands, rectal bleeding, or menstruation).
- To ensure the integrity of the sample, the laboratory must receive the patient specimens within 72 hours of collection. Patients should send stool samples to the laboratory according to the instructions stated in the *Cologuard* Patient Guide.
- Patients should be advised of the caution listed in the *Cologuard* Patient Guide. Patients should NOT drink the preservative liquid.
- The risks related to using the *Cologuard* Collection Kit are low, with no serious adverse events reported among people in a clinical trial. Patients should be careful when opening and closing the lids to avoid the risk of hand strain.

Summary and Explanation of the Test

Cologuard utilizes a multi-target approach to detect DNA and hemoglobin markers associated with colorectal cancer (CRC), as well as pre-malignant colorectal neoplasia. Three independent categories of biomarkers are targeted and provide an additive association with CRC and pre-malignant neoplasms.

The first category of biomarkers detects epigenetic DNA changes characterized by aberrant gene promoter region methylation. The specific methylated gene targets include N-Myc Downstream-Regulated Gene 4 (*NDRG4*) and Bone Morphogenetic Protein 3 (*BMP3*).[6,7] *NDRG4* and *BMP3* have been shown to be hypermethylated in colorectal cancer.[5,6] The *Cologuard* procedure incorporates bisulfite conversion of non-methylated cytosine residues to uracil in the DNA sequence to enable sensitive detection of hypermethylated *NDRG4* and *BMP3*.

The second category targets specific DNA point mutations in the v-Ki-ras2 Kirsten rat sarcoma

viral oncogene homolog (*KRAS*) gene, which encodes a small GTPase that is activated transiently as a response to extracellular stimuli or signals.[8,9,10] *KRAS* mutations have been detected in up to 35% of colorectal cancers and the 7 mutations in Exon 2 detected by *Cologuard* account for 98% of these *KRAS* mutations.[12] *KRAS* mutations, along with *NDRG4* and *BMP3* methylation markers, have been shown to be detected in the stool of subjects with colorectal neoplasia, including subjects with colorectal cancer and pre-malignant lesions.[4,11]

The third category of biomarker is non-DNA based and detects hemoglobin, which can be associated with colonic bleeding. Results from the methylation, mutation, and hemoglobin assays are integrated by the Exact Sciences Analysis Software to determine a Positive or Negative reportable result or invalid result.

Principles of the Procedure

Cologuard is designed to analyze patients' stool for the presence of DNA and hemoglobin markers, which may indicate the presence of colorectal cancer or pre-cancerous lesions. Patients use the *Cologuard* Collection Kit, consisting of a Container for collection of stool for DNA testing and a separate sampler (Tube) for collection of stool for hemoglobin testing. Both of these stool samples are required to obtain a *Cologuard* result.

In the processing procedure for DNA testing, the stool sample is mixed with buffer in the Container using the Sample Mixer. An aliquot of the buffered stool sample is centrifuged to pellet solids and generate supernatant. The assay procedure begins with treatment of the supernatant with an Inhibitor Removal Tablet to remove inhibitors that may affect the detection of the DNA biomarkers. Treated supernatant is then combined with denaturing reagents and incubated with target-specific magnetic particles using the Capture Incubator instrument to capture sequences for *NDRG4*, *BMP3*, *KRAS,* and *ACTB* (reference gene).

Using automated processes in the Capture Aspirator and Hamilton Microlab® STARlet (STARlet) instruments, targeted sequences are separated from the solution, washed, and eluted from the particles. Eluted DNA is split to provide two separate DNA aliquots for performing the methylation and mutation assays. The aliquot for the methylation assay is treated with bisulfite conversion reagents. Both aliquots are further purified with silica-coated magnetic beads from which DNA is eluted.

The Quantitative Allele-specific Real-time Target and Signal Amplification (*QuARTS*™) technology combines real-time PCR and invasive cleavage to perform allele-specific amplification and detection of methylated target DNA (*NDRG4*, *BMP3*) and specific DNA point mutations (*KRAS*) in the molecular assays. Each purified DNA aliquot is mixed with the appropriate *QuARTS* reaction master mix. The bisulfite-converted DNA is mixed with a master mix for the *NDRG4*, *BMP3*, and A*CTB QuARTS* reaction. The unconverted, purified DNA for *KRAS* detection is mixed with a master mix for the 7 *KRAS* mutations and *ACTB*. Both *QuARTS* reactions are processed using a real-time cycler in the same assay plate with the same cycling and detection program. Each assay for the *NDRG4*, *BMP3*, *ACTB*, and *KRAS* markers is monitored separately through an independent fluorescent detection channel.

In a parallel workflow, the hemoglobin assay stool sample is prepared and analyzed in a quantitative Enzyme-Linked Immunosorbent Assay (ELISA) that determines the concentration of hemoglobin in the sample. Each sample is incubated in a single well of a 96-well plate coated

with anti-hemoglobin antibody, which is then washed to remove any unbound material. A second anti-hemoglobin antibody conjugated to the enzyme horseradish peroxidase (HRP) is then added to the wells and incubated with a colorimetric substrate for HRP. After the reaction is stopped and the optical density read on a plate reader, the level of hemoglobin present in the stool sample is calculated using a calibration curve prepared from a set of calibrators with known hemoglobin concentrations.

Run control samples for both the molecular assays and hemoglobin assay are tested along with patient samples to show that the process has been performed appropriately. Run controls from the *Cologuard* DNA Control Kit (Exact Sciences, 100074) and *Cologuard* Hemoglobin Control Kit (Exact Sciences, 100073) are required in each run to obtain valid assay results. Results from the methylation, mutation, and hemoglobin assays are integrated by the Exact Sciences Analysis Software to determine a Positive or Negative reportable result or an Invalid result.

Reagents

Cologuard utilizes several reagent kits stored at different temperatures including DNA Capture Reagents (2 to 8°C), DNA Preparation Reagents (15 to 30°C), *QuARTS* Assay Reagents (-25 to -15°C), and Hemoglobin Assay Reagents (2 to 8°C).

Lots of reagents are matched for performing the assay. A Supplemental Lot Information sheet is supplied with the reagents. On the sheet is a 2D barcode or set of barcodes, the Supplemental Lot Information Barcode (SLIB), which includes calibration and lot matching information for that lot of reagents. The SLIB is scanned into the Exact Sciences Analysis Software prior to performing any portion of the automated run using these reagents.

Use lot numbers of reagents and calibrators listed in Supplemental Lot Information together. DO NOT mix or substitute reagents from Supplemental Lot Information containing different lot groupings.

Ancillary and bulk assay reagents (stored at 15 to 30°C) are also required to run *Cologuard*. Bulk assay reagents are not lot matched to *Cologuard* reagents and may be used with any lot of reagent kits.

DNA Capture Reagents (Exact Sciences, 100028)

Part #	Component	Description	Amount	# provided
200150	CAP BDS, Capture Beads	Magnetic particles with covalently bound oligonucleotide probes	7 mL	10

DNA Preparation Reagents (Exact Sciences, 100029)

Part #	Component	Description	Amount	# provided
200123	DEN SLN, Denaturation Solution	0.1 M NaOH solution	14.5 mL	10
200124	BIS SLN, Bisulfite Conversion Solution	Ammonium bisulfite solution	8.5 mL	10

200125	NEU SLN, Neutralization Solution	Potassium Acetate solution	8.5 mL	10
200222	DES SLN, Desulphonation Solution (Concentrate)	0.35 M NaOH solution	7.5 mL	10
200127	BND BDS, Binding Beads	Magnetic silica particles	8.4 mL	10
200218	DNA and *QuARTS* Supplementary Lot Information	n/a	1 each	1

QuARTS Assay Reagents (Exact Sciences, 100030)

Part #	Component	Description	Amount	# provided
200235	CAR SLN, Carrier Solution	Bovine Serum Albumin, Tris, EDTA	1600 µL	10
200130	ELU BFR, Elution Buffer	Tris, EDTA Solution	12.5 mL	10
200131	MIX A, Oligo Mix A, Methylation	Oligonucleotides, FRET probes, dNTPs	1200 µL	10
200132	MIX B, Oligo Mix B, Mutation	Oligonucleotides, FRET probes, dNTPs	1200 µL	10
200133	ENZ, Enzyme Mix	Enzymes in a buffer with glycerol	250 µL	10
200134	D CAL 1, DNA Calibrator 1, High Methylation	NDRG4, BMP3, ACTB DNA in buffer with non-human DNA carrier	60 µL	10
200135	D CAL 2, DNA Calibrator 2, Low Methylation	NDRG4, BMP3, ACTB DNA in buffer with non-human DNA carrier	60 µL	10
200136	D CAL 3, DNA Calibrator 3, High Mutation	KRAS, ACTB DNA in buffer with non-human DNA carrier	60 µL	10
200137	D CAL 4, DNA Calibrator 4, Low Mutation	KRAS, ACTB DNA in buffer with non-human DNA carrier	60 µL	10

Cologuard DNA Control Kit (Exact Sciences, 100074)

Part #	Component	Description	Amount	# provided
200139	DNA Control 1, High	Oligonucleotides, Tris, EDTA with Carrier DNA	15 mL	10
200140	DNA Control 2, Low	Oligonucleotides, Tris, EDTA with Carrier DNA	15 mL	10
200141	DNA Control 3, Negative	Oligonucleotides, Tris, EDTA with Carrier DNA	15 mL	10
200315	DNA Control Kit Supplementary Lot Information Barcode	n/a	1 each	1

Hemoglobin Assay Reagents (Exact Sciences, 100031)

Part #	Component	Description	Amount	# provided
200142	Hb PLATE, Hemoglobin Assay Plate	Mouse anti-Human Hemoglobin Antibody coated plate	1 plate	5
200143	SMP BFR, Sample Buffer	Tris, NaCl, casein	12 mL	5
200144	CONJ, Antibody Conjugate	Mouse anti-Human Hemoglobin Antibody-HRP Conjugate	12 mL	5

200100	SUBS, Substrate	Tetramethylbenzidine in buffer	12 mL	5
200101	STP SLN, Stop Solution	Acidic Buffered Solution	12 mL	5
200146	Hb CAL, Hemoglobin Assay Calibrator	Human Hemoglobin, buffer (lyophilized)	1 each	5
200219	Hemoglobin Assay Supplementary Lot Information	n/a	1 each	1

Cologuard Hemoglobin Control Kit (Exact Sciences, 100073)

Part #	Component	Description	Amount	# provided
200147	Hemoglobin Control 1, High	Human Hemoglobin, buffer (lyophilized)	1 each	5
200148	Hemoglobin Control 2, Low	Human Hemoglobin, buffer (lyophilized)	1 each	5
200149	Hemoglobin Control 3, Negative	Human Hemoglobin, buffer (lyophilized)	1 each	5
20031e	Hemoglobin Control Kit Supplementary Lot Information Barcode	n/a	1 each	1

Ancillary Materials and Bulk Assay Reagents

Required ancillary materials, kits and bulk assay reagents are available using the part numbers listed below.

Part #	Component	Description	Amount per vessel
200204	STL BFR, Stool Buffer	Tris, EDTA Solution	20 L
200151	TABLT, Inhibitor Removal Tablet	Polyvinylpolypyrrolidone with excipient	95 ea
200138	FILT, Spin Filter	Spin filters for 50 mL tubes	46 ea
200152	TUBES, Barcoded Mixing Tubes	Empty barcoded tubes	50 ea
200120	PRE WSH, Capture Bead Pre-wash	Sodium Bicarbonate Buffer	350 mL
200121	CAP SLN, Capture Solution	Guanidine Thiocyanate	450 mL
200122	CAP WSH, Capture Wash	MOPS Buffer, NaCl	450 mL
200126	BND SLN, Binding Solution	Guanidine Hydrochloride	450 mL
200129	CNV WSH, Conversion Wash Concentrate	Tris Buffer	200 mL
200145	Hb WSH, Hemoglobin Assay Wash Concentrate	Phosphate Buffer with detergent	300 mL

Procedural Warnings and Precautions

Warnings and notes emphasize important reagent information and critical instructions for safely performing laboratory procedures.

- Use standard laboratory precautions in accordance with applicable federal, state, and local regulations.
- Laboratory areas should be cleaned and maintained according to good laboratory practices for clinical laboratories processing biological specimens. Appropriate

procedures shall be defined by the laboratory director.

- Sodium hypochlorite may not be appropriate for decontamination of instruments and pipettes.
- Refer to user's manuals for complete decontamination procedures for instruments and equipment.
- Product components (residual product, packaging, waste) can be considered laboratory waste. Dispose in accordance with applicable federal, state, and local regulations.

Reagent Warnings and Precautions

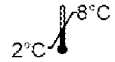
IVD	For *In vitro* diagnostic use
	Users should familiarize themselves with the instructions contained in the *Cologuard* Patient Guide, this booklet and the equipment used to perform the *Cologuard* prior to use.
	Caution: Patients should avoid bringing preservative solution in contact with skin or eyes. Irritation could result.
	Some reagents or waste are potentially corrosive or flammable. Dispose of all reagents in accordance with local, state, and Federal regulations. (CLSI doc GP5-A2, EPA/530-SW-86-014)
	Warning, biological hazard. Specimens may be infectious. Only personnel adequately trained in handling infectious materials should be permitted to perform this diagnostic procedure. Human materials used in Hemoglobin Assay Calibrator were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. As an additional precaution, Hemoglobin Assay Calibrator (Hb CAL) should be treated as potentially infectious. Dispose of all potentially biohazardous materials in accordance with local, state and Federal regulations.
	Danger, Corrosive. Skin and respiratory Irritant. Avoid contact with Denaturation Solution (DEN SLN), Bisulfite Conversion Solution (BIS SLN), and Desulphonation Solution (DES SLN) with skin, eyes, and mucous membranes. If these fluids come into contact with skin or eyes, wash with water. If spills of these fluids occur, dilute with water before wiping dry.
	Warning, Irritant. Avoid contact with Binding Solution (BND SLN), Capture Solution (CAP SLN) and Stop Solution (STP SLN) with skin, eyes and mucous membranes. If these fluids come into contact with skin or eyes, wash with water. If swallowed, DO NOT induce vomiting unless directed by poison control center. If spills of these fluids occur, dilute with water before wiping dry.
	Warning, Respiratory irritant. Avoid contact with Bisulfite Conversion Solution (BIS SLN) with skin, eyes and mucous membranes. If these fluids come into contact with skin or eyes, wash with water. If spills of these fluids occur, dilute with water before wiping dry. If swallowed, DO NOT induce vomiting unless directed by poison control center. If inhaled, move to fresh air. If breathing becomes difficult, give oxygen and consult physician.
	The Antibody Conjugate (CONJ) may contain a mixture of 5-chloro-2-metyhl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one which are components of ProClin®. The components are classified per applicable European Community (EC) Directives as: Irritants (Xi). Avoid contact with skin eyes and mucous membranes.
	Warning. Do not use sodium hypochlorite (bleach) to decontaminate surfaces or dispose of waste from steps using Bisulfite Solution (BIS SLN) or Capture Solution (CAP SLN). Salts from these reagents are not compatible with cleaning solutions containing bleach.

Instrument Warnings and Precautions

	Users should familiarize themselves with detailed user information contained in equipment user manuals prior to following the *Cologuard* laboratory procedure.
	Conduct instrument maintenance according to individual instrument user's manuals on all instruments to ensure safe and appropriate use.
	Conduct STARlet daily and weekly maintenance as required. Failure to empty liquid waste may result in release of hazardous materials into the environment. Failure to empty tip waste may result in an aborted run and/or contamination of the instrument deck.
	There are environmental specifications for Hamilton STARlet that are specific to Cologuard. Ensure that instruments are maintained in environments at 18-26 C with 20-85% RH.

Reagent Storage and Handling Requirements

Part #	Reagent Group	Storage Requirement	Additional Handling Requirements
100028	DNA Capture Reagents	2°C–8°C	DO NOT FREEZE CAP BDS.
100029	DNA Preparation Reagents	15°C–30°C	See Warnings and Precautions specified for DEN SLN, BIS SLN, and DES SLN. Add 17.5 mL 100% isopropanol to DES SLN concentrate before use. Store BIS SLN protected from light.
100030	*QuARTS* Assay Reagents	-25°C–-15°C	Reagents may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.
100031	Hemoglobin Assay Reagents	2°C–8°C	See Warnings and Precautions specified for STP SLN.
200204	STL BFR, Stool Buffer	15°C–30°C	
200151	TABLT, Inhibitor Removal Tablet	15°C–30°C	
200138	FILT, Spin Filter	15°C–30°C	
200152	TUBES, Barcoded Mixing Tubes	15°C–30°C	
200120	PRE WSH, Capture Bead Pre-wash	15°C–30°C	
200121	CAP SLN, Capture Solution	15°C–30°C	See Warnings and Precautions specified for CAP SLN. If precipitate is observed, heat at 35°C to 50°C to solubilize.
200122	CAP WSH, Capture Wash	15°C–30°C	
200126	BND SLN, Binding Solution	15°C–30°C	If precipitate is observed, heat at 35°C to 50°C to solubilize.
200129	CNV WSH, Conversion Wash Concentrate	15°C–30°C	Prepare working solution before use according to instructions in the *Cologuard* Laboratory Procedure.

Part #	Reagent Group	Storage Requirement	Additional Handling Requirements
200145	Hb WSH, Hemoglobin Assay Wash Concentrate		Prepare working solution before use according to instructions in the *Cologuard* Laboratory Procedure. If precipitate is observed in concentrate, heat at 35°C to 50°C to solubilize.

Instrumentation

Part #	Instrument	Manufacturer (Supplier)
300810	Sample Mixer	Exact Sciences
11675200	MaxQ™ 2000 Open-Air Platform Shaker	Thermo Fisher
300551	Capture Shaker Rack	Exact Sciences
300546	Capture Incubator	Exact Sciences
100034	Capture Aspirator	Exact Sciences
100065	Hamilton Microlab® STARlet	Hamilton (Exact Sciences)
100066	STARlet Hemoglobin Package	Exact Sciences
4406984	7500 Fast Dx IVD w/laptop	Life Technologies
ELx808™	ELx808 Custom Plate Reader	BioTek
3100620	620 nm Filter assembly for ELx808	BioTek
200268	System Computer	Exact Sciences

The primary instruments required to perform the laboratory procedure for *Cologuard* are listed above. These instruments and supporting software are provided and installed separately through Exact Sciences service prior to training of laboratory personnel. The Cologuard *Laboratory Procedure* section outlines the specific use of these instruments for performing *Cologuard*.

The Sample Mixer is used to mix the stool sample for DNA testing (Container) received from the patient (refer to Cologuard *Laboratory Procedure, Preparation of Stool Homogenate*). The MaxQ™ Shaker is equipped with the Capture Shaker Rack for use in preparing the supernatant for the DNA Capture (refer to Cologuard *Laboratory Procedure, DNA Capture*). The Capture Incubator and Capture Aspirator are equipment used during the DNA Capture steps of the assay (refer to Cologuard *Laboratory Procedure, DNA Capture*). The Capture Incubator performs sample heating, cooling and mixing of 50 mL tubes. The Capture Aspirator automates magnetic particle separation from supernatant in a 50 mL tube format.

Once DNA Capture steps are completed, the Hamilton Microlab® STARlet is used for automated DNA preparation and *QuARTS* plate setup as well as automated Hemoglobin plate setup (refer to Cologuard *Laboratory Procedure, DNA Preparation and* QuARTS *Assay* and *Hemoglobin Assay*). The 7500 Fast Dx instrument is used to perform the QuARTS reactions setup in the prepared *QuARTS* assay plate. The ELx808™ Custom Plate Reader is used to measure hemoglobin assay plate.

Specimen Collection and Preparation for Analysis

Specimens for use with *Cologuard* must be collected with the *Cologuard* Collection Kit (Exact Sciences, 100026), including a stool sample for DNA testing (Container) and a stool sample for Hemoglobin testing (Tube). Detailed instructions for sample receipt and processing are outlined in the *Cologuard* Laboratory procedure below. Known interfering substances that may impact the assay results are summarized in the *Performance Characteristics, Interfering Substances* section below.

	Patients should familiarize themselves with detailed information contained in *Cologuard* Patient Guide and collection instructions before completing sample collection.
	Stool samples must be collected with the *Cologuard* Collection Kit (Exact Sciences, 100026).
	The *Cologuard* Collection Kit should be stored protected from direct sunlight at ambient temperature.
	Patients should not provide a sample if they have diarrhea or blood in their urine or stool from bleeding hemorrhoids, bleeding cuts or wounds on their hands, rectal bleeding, or menstruation.
	To ensure the integrity of the sample, the laboratory must receive patient specimens within 72 hours of collection. Detailed instructions are outlined in the *Cologuard* Laboratory Procedure.
	Samples may be stored by the laboratory until processing. The Tube (hemoglobin sample) can be stored for up to 7 days after receipt at 2 to 8°C. The Container (DNA sample) can be stored ambiently or at 2 to 8°C and should be processed within 6 days of collection. Detailed processing instructions are outlined in the *Cologuard* Laboratory Procedure.
	Avoid cross-contamination during the specimen handling steps. If gloves come into contact with specimen, change gloves to avoid cross-contamination.

Cologuard Laboratory Procedure

Receipt of *Cologuard* Collection Kit

The patient collects a stool sample using the *Cologuard* Collection Kit (Exact Sciences, 100026). Stool samples are sent to the laboratory according to the *Cologuard* Patient Guide that accompanies the *Cologuard* Collection Kit. Laboratory processing begins with receipt of the collection kit and preparation of stool for DNA capture.

1. Check that both the Tube (hemoglobin sample) and Container (DNA sample) are present and review the information noted on the handwritten labels affixed by the patient.
   a. Confirm that collection date and time occurred less than 72 hours prior to receipt.
2. Remove the samples and discard packaging in accordance with local regulations.
3. Vortex hemoglobin sample tube at highest speed until grooves of the probe are void of stool.
4. Store the samples appropriately until processing:
   a. The hemoglobin sample can be stored for up to 7 days after receipt at 2 to 8°C.
   b. The DNA sample can be stored at ambient temperature or at 2 to 8°C and should be processed within 6 days of collection.

Preparation of Stool Homogenate for DNA Testing

1. Weigh the Container (containing sample) and record the Sample Weight.
2. Calculate Stool Weight:

a. Stool Weight = Container (g) – 535 g (empty container + preservation weight).

3. Based on the calculated Stool Weight, adjust the stool: buffer ratio as follows:
   b. If stool weight is less than or equal to 0 g, sample is invalid. Discard sample and request a replacement.
   c. If stool weight is greater than 0 g and less than or equal to 72 g, proceed to Step 4.
   d. If stool weight is greater than 72 g and less than 300 g, calculate amount of Stool Buffer (Exact Sciences, 200204) to add. Stool Buffer can be added by volume **or** by weight (see table below). Open the Container and add the Stool Buffer. Proceed to Step 4.
   e. If stool weight is greater than or equal to 300 g, the sample is invalid. Discard sample and request a replacement.

Stool Weight (X)	Stool Buffer to Add*		Additional Information
	By Volume (mL)	By Weight (g)	
X ≤ 0 g	N/A	N/A	Invalid sample
0 g < X ≤ 72 g	N/A	N/A	Sample adequately buffered
72 g < X ≤ 280 g	4X – 290	1.04(4X – 290)	Dilution yields 1 g stool per 4 mL buffer
280 g < X < 300 g	(1143 – X)/1.04	1143 – X	Dilution maximized based on capacity of Container
X ≥ 300 g	N/A	N/A	Invalid Sample
*Stool Buffer may be added by volume or by weight. The density of the buffer (1.04 g/mL) is used in conversions between volume and weight.			

4. Tighten the Container lid following instructions in the Sample Mixer User's Manual.
5. Place the Container in the Sample Mixer (Exact Sciences, 300810), secure the container with the lid attachment, and close the mixer door. Initiate the mixing cycle.
6. When the mixing is complete, remove the Container from the mixer, and then remove the lid from the Container.
7. Prepare at least two 50 mL tubes (Corning, 430829) with barcoded labels to identify the sample.
8. Transfer homogenate sample up to the 45 mL graduation mark to each of the 50 mL tubes.
9. Place samples at -15°C or colder for at least 8 hours prior to use. Discard the processing pipette, any remaining homogenate, and the Container according to local regulations.

Assay Overview

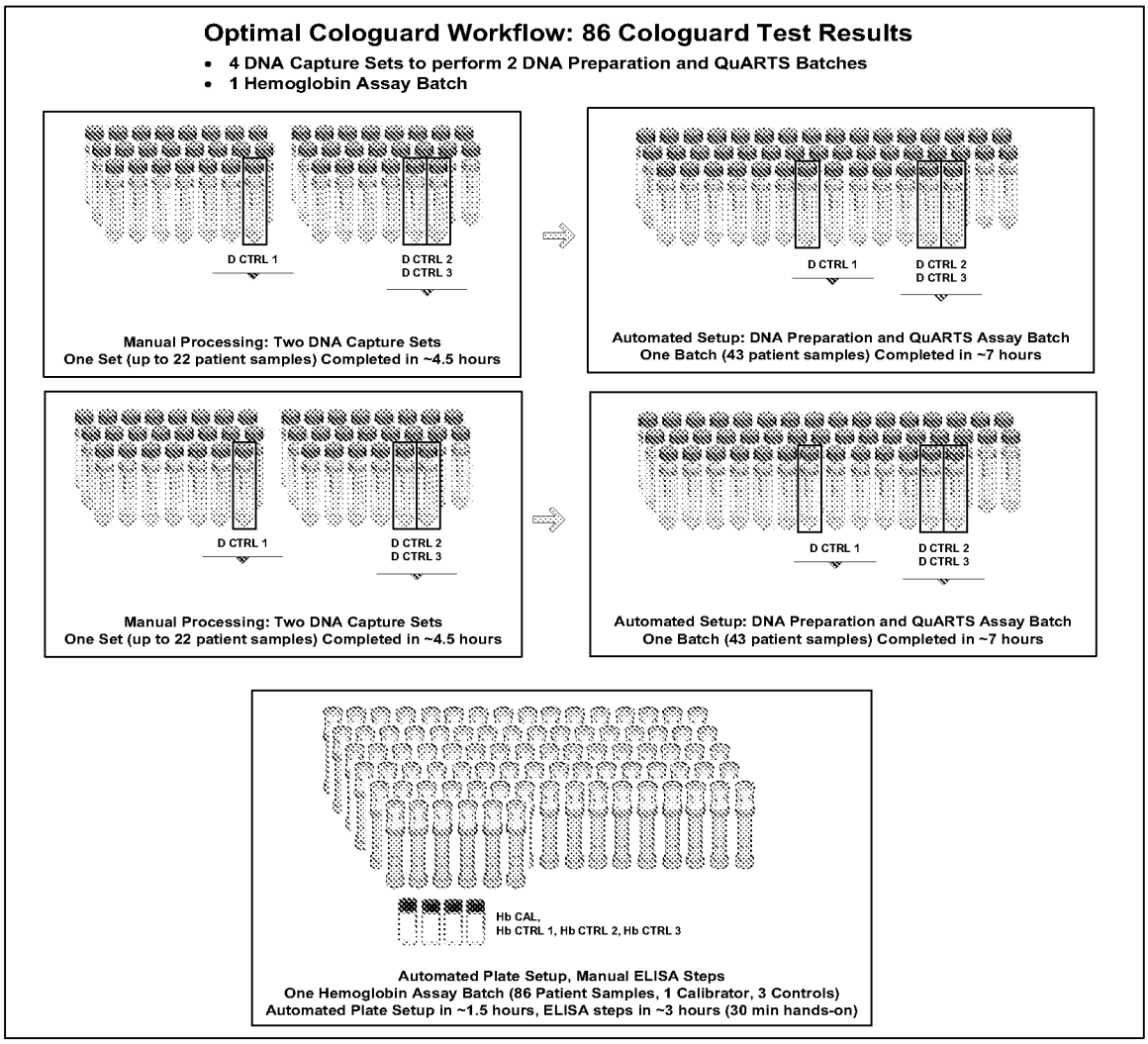
Each *Cologuard* reagent kit contains sufficient materials for 480 tests. This includes reagents for 5 groups of 86 patient samples and the required controls and calibrators. The assay procedure includes steps for DNA Capture, DNA Preparation, *QuARTS* Assay, Hemoglobin Assay, and Data Analysis using the Exact Sciences System Software. DNA Capture steps are performed manually and are typically processed in sets of 23 patient and control samples. DNA Preparation and *QuARTS* Assay steps are performed using the Microlab® STARlet (STARlet), custom built for Exact Sciences, and are processed in batches of up to 46 samples, including 43 patient samples and the required controls. Hemoglobin Assay steps are performed in 96-well assay plates and typically include batches of up to 86 patient samples and required calibrators and controls. This assay uses the STARlet for plate setup, followed by a manual sandwich

ELISA. Optimal usage of *Cologuard* is achieved with four full sets of DNA Capture, two full batches of DNA Preparation and *QuARTS* Assay, and one full batch for the Hemoglobin Assay.

DNA and Hemoglobin Control Samples are supplied in the *Cologuard* DNA Control Kit (Exact Sciences, 100074) and the *Cologuard* Hemoglobin Control Kit (Exact Sciences, 100073). Controls D CTRL 1, D CTRL 2, and D CTRL 3 are required for each batch of DNA Preparation and *QuARTS* assay samples processed on the STARlet. At least one positive DNA Control (D CTRL 1 or D CTRL 2) is required for every distinct set of DNA Capture tubes. Hemoglobin Controls (Hb CTRL 1, Hb CTRL 2, and Hb CTRL 3) are required for each plate of Hemoglobin Assay samples.

Reagents not denoted as ancillary material or bulk assay reagents are packaged for single use and the leftover reagents cannot be reused. Test samples should be stored and run in maximum batch sizes as defined in the "Optimal *Cologuard* Workflow" figure below to maximize use of the reagents. Each DNA Capture set **must** contain at least one positive control for every run performed, regardless of the number of test samples, and each DNA Preparation and *QuARTS* assay must contain all DNA controls. Hemoglobin Assay runs must contain all controls. An example of optimal setup is shown below.

Example Cologuard Setup



Prepare Capture Beads

NOTE: For each DNA Capture set of 23 tubes, 3.25 mL of prepared Capture Beads are required. If desired, multiple tubes of Capture Beads from a single lot can be prepared simultaneously for processing additional sets.

1. Set the Capture Incubator (Exact Sciences, 300546) to preheat ("Bead Prep 1" program).
2. Allow Capture Beads (Exact Sciences, 200150) to sit at room temperature for a minimum of 30 minutes.
3. Vortex Capture Beads at highest setting for 30 seconds to suspend the beads.
4. Label the 50 mL conical tube(s) with Capture Bead preparation date and lot information.

Labels used in the Capture Incubator have specific requirements for size, material and thickness. Label the bead preparation tubes using permanent marker or refer to Capture Incubator User's Manual for detailed label specifications.

5. Transfer 3.25 mL of beads to the labeled 50 mL tube.
6. Add 10 mL of Capture Bead Pre-wash (Exact Sciences, 200120) and secure the 50 mL tube cap.
7. When the Capture Incubator has reached programmed temperature and display prompts user to insert test tubes, place tube(s) in the Capture Incubator. Close cover and press the 'Start/Select' button to proceed with the cycle.
8. When the cycle is complete, remove tube(s) from the incubator and place in the centrifuge with appropriate balance tube, if necessary. Centrifuge the tube(s) until the centrifuge reaches 500 × g for and hold for 1-10 seconds.
9. Remove cap(s) and transfer the tube(s) to the first row (left-most) of the Capture Aspirator (Exact Sciences, 300490). Execute the "Prep Beads" protocol to remove supernatant from the tube(s).

NOTE: If operator prefers to prepare greater than six tubes, see Procedural Notes and Precautions, Prepare Capture Beads (for >6 tubes).

10. When aspiration run is complete, remove tube(s) from Capture Aspirator and add 3.25 mL of fresh Capture Bead Pre-wash solution to each tube(s), replace cap(s) and vortex at highest setting until all beads are suspended.

NOTE: Once the Capture Beads have been prepared, they can be stored in closed tube for up to 7 days at 2-8°C before use.

Prepare Samples and Perform DNA Capture

Prepare and Label Sample Tubes

1. Remove stool aliquot samples and DNA controls (D CTRL 1-3) from storage.
   a. Place frozen samples in racks to allow air to circulate around the tubes. Leave racks of frozen samples at 2 to 8°C for at least 13 hours, but no more than 80 hours, until use.

b. Equilibrate DNA controls at room temperature for at least 30 minutes before further processing.

2. Create three labels for subsequent processing steps for each tube. These steps will include Inhibitor Removal Tablet addition (denoted as "TAB" or equivalent), use of Spin Filter (denoted as "SPN" or equivalent), and Capture Incubation (denoted as "CAP" or equivalent).

Prepare Supernatant

NOTE: Ensure that an aliquot of Stool Buffer (Exact Sciences, 200204) is available for use in subsequent steps if needed for possible volume adjustment.

1. Centrifuge the stool sample aliquots for 45 minutes at a setting of 4500 × g. Ensure that the centrifuge is balanced.
2. When the centrifugation is complete, promptly and carefully remove the tubes and place in racks.

NOTE: If the interface between pellet and supernatant becomes obviously disrupted (e.g., tube is dropped or inverted), repeat Steps 1-2.

3. Confirm that the labels from the centrifuged stool sample aliquots and DNA control tubes match the labels on the prepared, clean tubes for the next step.
4. Add one Inhibitor Removal Tablet (Exact Sciences, 200151) to each "TAB" tube before transferring samples.
5. Transfer 14 mL of supernatant from the spun stool sample aliquots and the DNA control tubes into the respective, clean, labeled "TAB" tubes.

NOTE: Ensure that only clean tube caps are used and that appropriate steps are taken during sample transfer to minimize any risk of cross-contamination. Do not interchange caps between tubes once the caps have been exposed to a sample.

NOTE: Aspirate centrifuged stool samples slowly and avoid disturbing the solid/liquid interface. Avoid aspirating any material from the pellet or material floating on the surface of the supernatant.

   a. If the volume of the supernatant is between 5 mL and 14 mL, bring the total volume of supernatant to 14 mL with Stool Buffer (Exact Sciences, 200204).
   b. If the volume of the supernatant is less than 5 mL, store the supernatant at 2 to 8°C until it can be combined with more supernatant obtained by repeating Steps 1 – 5 with additional homogenate aliquots as necessary.

6. Transfer the capped tubes to the MaxQ Shaker (Shaker) (Thermo Fisher, 11675200) with the Capture Shaker Rack (Exact Sciences, 300551) and mix for 15 minutes at 400 RPM.
7. After this point, the used 50 mL tubes with stool pellet may be discarded according to local regulations.
8. After mixing sample supernatants with the Inhibitor Removal Tablet, confirm that the labels from these tubes match the labels on the prepared, clean "SPN" tubes for the next step.
9. Place one Spin Filter (Exact Sciences, 200138) into each "SPN" labeled tube before transferring samples from Step 6. Reserve tube caps for use in a future step.
10. Swirl each tube from Step 6 to suspend content, remove cap and pour the contents into the spin filter of the respectively "SPN" labeled spin filter tube. Close the lid of the spin filter. Repeat for all samples.
11. After this point, the used "TAB" tube and cap may be discarded according to local

regulations.

12. Once all samples are transferred to spin filters, place spin filter tubes into the centrifuge. Ensure that centrifuge is balanced and spin for 6 min at 3300 × g.
13. Remove the tubes from the centrifuge and confirm that the labels from these tubes match the labels on the prepared, clean "CAP" tubes for the next step.
14. For each tube, remove the spin filter from the tube, and then transfer 10 mL of supernatant to the capture tube ("CAP" label).
15. Confirm that the transferred supernatant contains 10 mL volume.
    a. If a tube contains between 5 and 10 mL supernatant, bring the volume up to 10 mL with Stool Buffer (Exact Sciences, 200204).
    b. If a tube contains less than 5 mL supernatant, store the filtered supernatant at 2 to 8°C until it can be combined with extra supernatant obtained by repeating Steps 1-14 with additional homogenate aliquots, as necessary.
16. At this point, the used 50 mL tubes with spin filters may be discarded according to local regulations.
17. Proceed to next step or store at 2-8°C for up to 6 days.

Capture Incubation

NOTE: If prepared supernatant was stored at 2-8°C, incubate at 15-30°C for 30 minutes.

NOTE: If applicable, remove prepared Capture Beads from 2-8°C and incubate at 15-30°C for 30 minutes before use.

1. Inspect Capture Solution (Exact Sciences, 200121) for precipitate. If precipitation is present, warm at 35°C-50°C for 20 minutes or until solubilized. Invert to mix as needed.
2. Add 7.25 mL of Capture Solution to each capture tube, ensuring that the Capture Solution runs down the inside of the tube to avoid foaming.

NOTE: Prepared Capture Beads must match the reagent lots listed on the DNA and* QuARTS *Supplementary Lot Information (Exact Sciences, 200218) that will be used for the assay run.

3. Vortex prepared Capture Beads for 30 seconds at the highest setting to suspend the beads.

If Capture Beads are not suspended before transfer, DNA Capture may not work properly.

4. Add 125 µL of beads to each of the capture tubes and then tighten the tube caps.
5. Place all tubes into the Capture Incubator using the Capture Incubator Tube Lift (Exact Sciences, 300547) and then start the EXAS8 program.

NOTE: Place a 17.5 mL water-filled blank tube into each empty position of the Capture Incubator.

6. When the program reaches completion, remove tubes from the Capture Incubator, remove and discard caps, and place open tubes in the Capture Aspirator, 300490.
7. Perform capture aspiration using the "BIND 10 min" program.
8. Remove the tubes from the Capture Aspirator and inspect for complete aspiration.

NOTE: If incomplete aspiration is observed, bring tube volume to 10 mL using Capture Wash, mix by pulse vortexing and repeat the BIND 10 min program.

9. Promptly add 750 µL of Capture Wash (Exact Sciences, 200122) to each tube.

10. Cap the tubes using reserved caps from *Prepare Supernatant*, Step 9. Place tubes into the Shaker, and mix for 1 minute at 400 RPM. Confirm that the Capture Beads are suspended in each tube.

NOTE: If beads are not suspended, rotate tube and mix for 1 minute at 400 RPM.

11. Remove the tubes and store at 2 to 8°C if not proceeding to *Automated DNA Preparation and* QuARTS *Assay Setup* immediately. Closed tubes containing capture wash and beads can be stored for up to 4 days before use.

DNA Capture will need to be performed on two full sets (46 samples and controls total) to obtain a full batch of samples for the next steps.

DNA Preparation and *QuARTS* Assay

DNA Preparation and *QuARTS* Assay steps are processed in batches of up to 46 samples from the DNA Capture steps. Input samples include up to 43 patient samples in addition to D CTRL 1, D CTRL 2, and D CTRL 3 in each batch. DNA preparation and the *QuARTS* assay plate setup are only performed on the automated STARlet. The program for the Exact Sciences STARlet Interface Software guides the operator through loading the sample tubes, resources, and reagents onto the system.

The system uses barcodes to identify samples and reagents. The barcode on each sample tube is used to ensure tracking to the final result while the reagent barcode tracking ensures that the matched lots of reagents are used together and that the reagents have not expired. It is the responsibility of the assay operator to ensure that the Capture Beads used in the capture process match the reagent lots listed with DNA and *QuARTS* Supplementary Information (Exact Sciences, 200218) used for an assay run. Errors detected by the system are reported in the run results.

DNA and *QuARTS* Reagent Supplemental Lot Information (Exact Sciences, 200218) is used to transfer lot and calibrator information into the Exact Sciences System Software. The information needs to be entered only once for each unique Supplemental Lot Information lot number. Similarly, *Cologuard* DNA Control Kit Supplemental Lot Information (Exact Sciences, 200315) is used for transfer of the control values and acceptance limits for the particular kit lot of controls used in the procedure.

Detailed instructions are provided in the software screens on the correct positioning of each reagent and all consumables, samples, and racks. Each DNA Preparation and *QuARTS* batch consists of up to 43 patient samples and 3 controls. Users are instructed through the software to provide 2 calibrators for the methylation assay (D CAL 1 and D CAL 2 (Exact Sciences, 200134, 200135)), and 2 calibrators for the mutation assay (D CAL 3 and D CAL 4 (Exact Sciences, 200136, 200137)). Patient samples, controls, and calibrators for both methylation and mutation assays are set up in one 96-well *QuARTS* reaction plate. Additional instructions can be found in the Exact Sciences System Software User's Manual.

The steps for *Automated DNA Preparation and* QuARTS *Plate Setup* are completed in about 7 hours. After the DNA preparation steps are completed, the instrument prompts the user to mix, uncap, and replace reagents for the *QuARTS* plate setup. When the *QuARTS* plate is ready to run, the user removes the plate, covers with a plate seal, centrifuges to ensure the reagents are

at the bottom of all wells, and runs the plate on the 7500 Fast Dx Real-Time PCR Instrument (7500 Fast Dx; Life Technologies, 4406984). The *QuARTS* analytic run is completed in approximately 2.5 hours. Once complete, the data are exported to the Exact Sciences Analysis Software and the run results are calculated. Methylation and Mutation assay runs are considered valid if actual results from all DNA controls are within the expected ranges included in the *Cologuard* DNA Control Supplemental Lot Information, the calibration curve meets the acceptance criteria, and no fatal processing errors were detected by the system.

Automated DNA Preparation and *QuARTS* Assay Setup

Reagent Preparation

1. Assemble the following reagents. Equilibrate the Carrier Solution (Exact Sciences, 200235) to room temperature (may take up to 30 minutes). Steps 2-4 below may proceed while the Carrier Solution equilibrates.

Part #	Component Abreviation / Name
200122	CAP WSH, Capture Wash
200123	DEN SLN, Denaturation Solution
200124	BIS SLN, Bisulfite Conversion Solution
200125	NEU SLN, Neutralization Solution
200222	DES SLN, Desulphonation Solution
200127	BND BDS, Binding Beads
200126	BND SLN, Binding Solution
200129	CNV WSH, Conversion Wash
200235	CAR SLN, Carrier Solution

2. Add 17.5 mL of 100% isopropanol to the Desulphonation Solution (Exact Sciences, 200222) bottle, replace cap, and invert to mix 10 times.
3. To prepare Conversion Wash, add 800 mL of 100% ethanol to the Conversion Wash (Exact Sciences, 200129) bottle, replace cap, and invert to mix 10 times. Mark date on the prepared Conversion Wash bottle once ethanol is added. Prepared Conversion Wash can be used for up to 1 month.
4. Remove the captured DNA sample tubes resulting from *Capture Incubation* steps above from storage and allow samples to come to room temperature.
5. Place the tubes into the Shaker and mix for 1 minute at 400 RPM.

STARlet Setup

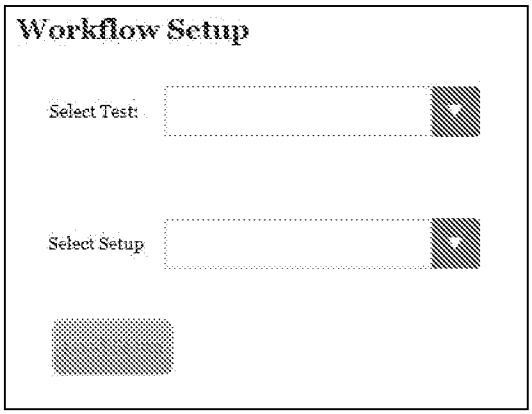
1. Check for outstanding maintenance at the beginning of each day prior to performing the run.
   a. Log into the Exact Sciences STARlet Interface Software.
   b. Under the Maintenance Monitor, see whether Maintenance Check is listed as 'valid' or 'invalid'. The monitor also lists the last date checked.
   c. If maintenance check is listed as 'valid', daily or weekly maintenance does not need to be performed.
   d. If maintenance check is listed as 'invalid', select the prompt to run maintenance.
   e. A new pop-up appears that lists both daily and weekly maintenance and when each was last performed.

f. Select the required maintenance type, select the green arrow to begin, and follow the software prompts to perform the maintenance.

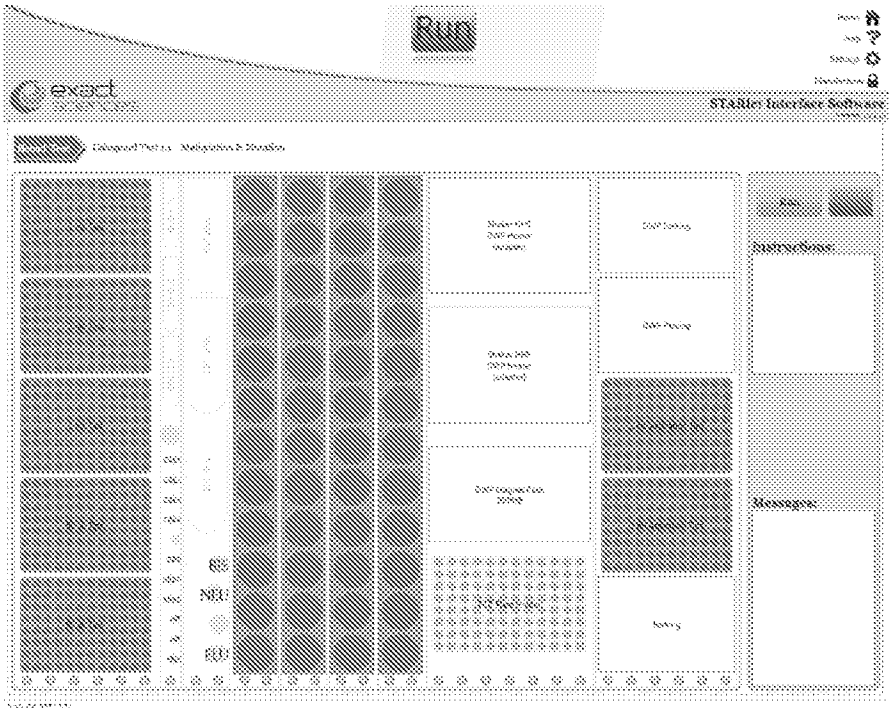
NOTE: Weekly maintenance covers all of the daily maintenance tasks. If weekly maintenance is being executed, daily maintenance does not need to be run.

NOTE: Cologuard methods will not begin if required maintenance has not been completed successfully.

2. If SLIBs have not been previously scanned into the Analysis Software, follow instructions for *Entering Supplementary Lot Information* under the *Procedural Notes and Precautions* section.
3. Select the *Cologuard* test and the Methylation & Mutation setup run on the Exact Sciences STARlet Interface Software, then select 'Load Setup' to initiate the run.



4. The deck layout diagram appears as shown below. Select 'Run' to start the loading process.



5. Confirm that the loading tray positions in front of the carriers on the deck are clear and hit 'Next' in order to prompt the instrument to unload any carriers stored in the instrument.

NOTE: Carriers are unloaded from the deck left to right and loaded right to left.

6. Load the appropriate carriers on the loading tray according to the deck layout. Load two deep-well plates (Axygen, P-DW-20-C) for Capture Wash and for conversion/cleanup and two trays 1000 µL CORE tips (Hamilton, 235905) in the right hand tip carrier.

Load only full trays of tips, or an invalid run may result.

Do not store any new or unused tips for waste aspiration in the right carrier between runs. Any tips present in the righthand tip carrier from a previous run must be presumed to be used and discarded to prevent possible aborted runs or cross-contamination of samples.

7. Load one MicroAmp Fast 96-well plate (Life Technologies, 436906) with barcode to the front.
8. Load uncapped samples and controls into 50 mL Tube Carriers (Hamilton, 182045) working back to front, left to right.
   a. Place samples in the sample carriers back to front, left to right, with no empty positions between tubes.
   b. Place controls in the sample carriers with no empty positions between the first and last sample or control.
   c. Place all sample carriers on deck, even if empty.

NOTE: Unread sample barcodes may require repositioning or manual barcode entry to correct the error. Results for samples with manually entered barcodes are flagged in the reports.

For the Methylation & Mutation method, three controls (D CTRL1, D CTRL 2, and D CTRL 3) must be present within the sample carriers for the run to begin.

Push each tube to the bottom of the rack and ensure that the barcode is visible in the slot on the right.

Empty positions are not permitted in sample carriers, except after the last loaded sample. Always load samples from left to right with no empty spaces between samples. Load all carriers regardless of the number of samples, placing empty carriers at the end.

Carriers with unread sample barcodes will be unloaded. The barcodes must be adjusted and the carrier reloaded until the barcode is successfully rescanned, or barcode sample IDs may be entered manually by the operator.

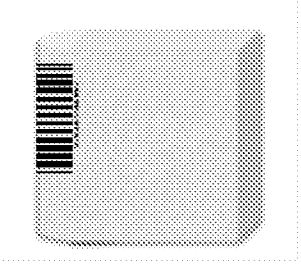
   d. When the sample carriers are successfully loaded, a prompt confirming the number of samples appears in the Instructions Box. Select 'Yes' if sample count is correct. Select 'No' if sample count is not correct.
   e. Selecting 'No' will unload the carriers for the operator to correct the issue. Once corrected, select 'Next'.

9. Load Reagents as shown in the on-screen deck layout into the indicated carrier positions.
   a. If a SLIB needs to be entered to continue setup of the run, scan the appropriate supplemental lot information barcodes using the 2D barcode scanner into the Analysis Software prior to hitting 'Next' to reload the carrier.
   b. See the table below for components and special instructions for individual reagents.

NOTE: Reagents are loaded and checked to match lot numbers from a SLIB scanned into the Analysis software. Reagents are identified and confirmed to be the correct lot and location by scanning their barcodes as they are loaded on the instrument. A run will not proceed with incorrect reagent part numbers or reagents from mixed SLIB lots (master lot mismatch) or reagents with unknown lot numbers.

NOTE: If any reagents are not recognized or do not match to a SLIB in the system, the carrier containing the mismatched reagents is unloaded. The software prompts the user to correct the issue and then select 'Next' to reload the carrier.

Transfer peel-off barcodes to troughs so that barcode is placed on the curved edge of the trough, starting at the top of the trough with the curved edge of trough on the left, as shown in the following figure. Human-readable barcode content should be perpendicular to the top of the trough as indicated below.



Part #	Component	Additional Instruction
200122	CAP WSH, Capture Wash	On first use, transfer peel-off barcode to clean 200 mL trough. Mix by inverting bottle 10 times, then transfer 100 mL of CAP WSH to trough. Trough is rinsed with distilled water after each use and allowed to dry, then re-used for the remaining volume of the CAP WSH bottle.
200123	DEN SLN, Denaturation Solution	Transfer peel-off barcode to clean 50 mL trough. Mix by inverting bottle10 times, then transfer all contents to trough.
200235	CAR SLN, Carrier Solution	Ensure liquid is completely thawed. Vortex 3-5 seconds at highest speed, spin briefly to collect volume, then **UNCAP** and place vial in the indicated carrier.
200124	BIS SLN, Bisulfite Conversion Solution	Mix by inverting 5 times, then **UNCAP** and place vial in the indicated carrier.
200125	NEU SLN, Neutralization Solution	Mix by inverting 10 times, then **UNCAP** and place vial in the indicated carrier.
200222	DES SLN, Desulphonation Solution (after isopropanol addition)	Transfer peel-off barcode to clean 50 mL trough. Transfer all contents to trough and cover with 50 mL Trough Lid (Exact Sciences, 100072).
200127	BND BDS, Binding Beads	Transfer peel-off barcode to clean 50 mL trough. Vortex bottle for 30 seconds, then transfer all contents to trough.
200126	BND SLN, Binding Solution	On first use, transfer peel-off barcode to clean 200 mL trough. Mix by inverting bottle 10 times, then transfer 100 mL to trough. Trough is rinsed with distilled water after each use and allowed to dry, then re-used the remaining volume of the BND SLN bottle.
200129	CNV WSH, Conversion Wash (after ethanol addition)	On first use, transfer peel-off barcode to clean 200 mL trough. Mix by inverting bottle 10 times, then transfer 200 mL to trough and cover with Trough Lid (Exact Sciences, 100071). Trough is rinsed with distilled water after each use and allowed to dry, then re-used for the remaining volume of the CNV WSH bottle.

Part #	Component	Additional Instruction
200130	ELU BFR, Elution Buffer	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200131	MIX A, Oligo Mix A, Methylation	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200132	MIX B, Oligo Mix B, Mutation	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200133	ENZ, Enzyme Mix	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200134	D CAL 1, DNA Calibrator 1, High Methylation	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200135	D CAL 2, DNA Calibrator 2, Low Methylation	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200136	D CAL 3, DNA Calibrator 3, High Mutation	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.
200137	D CAL 4, DNA Calibrator 4, Low Mutation	Place **CAPPED** tube onto the instrument deck. Cap will be removed at a later step.

10. Load two capped, barcoded empty tubes (Exact Sciences, 200152).
11. Load three trays 1000 µL CORE (Hamilton, 235905) and two trays 50 µL CORE (Hamilton, 235948) pipette tips into the left hand tip carrier.

Load only full trays of tips, or an invalid run may result.

12. When all carriers on the loading tray have been properly loaded with reagents, samples, and consumables, select 'Next'.
13. As carriers are loaded into the STARlet, a series of prompts will appear in the Instructions box, prompting the user to select tips, confirm sample counts, and correct reagent and sample loading issues such as mismatched lots or unread barcodes, if applicable. Select 'Next' after each action is performed.

NOTE: The system will check for the correct location or lot (when applicable) of tips, reagents, or samples as they are loaded by scanning and recording the item barcodes. Items with unread barcodes are indicated in red on the deck layout graphic.

14. When loading tips, Tip inventory should be updated when the tip tray becomes highlighted by a blinking red box on the screen.
    a. To update the tip count, select each tip tray so that the larger image appears, then select the blue +/- at the top of each column to auto-fill that column or select 'New Tray' to fill the entire tray with tips.
    b. A blue filled circle indicates that the user has verified that a tip is present in the corresponding tray location.
    c. Select 'Done' when the on-screen inventory matches the loaded tray.

NOTE: Ensure that all tip trays placed on the deck are completely filled and tip inventory is updated. Running without full trays of tips may lead to invalid assay results. If consolidating tips into a tray, make sure tip type matches tip barcode.

DNA Preparation

1. After all required reagents, samples, and controls have been loaded, the automated method begins. The STARlet records the plate barcode as the run identifier.
2. During the method, liquid transfer verification is used by the software to monitor the transfer of reagents and samples. Errors detected by the system are reported in the run results.
3. The Messages box displays status notifications during the run, such as approximate end time of incubation steps.

QuARTS Plate Setup

Plan time and resources accordingly. Once the *QuARTS* Plate Setup is complete (Steps 1-7 below), the run on the 7500 Fast Dx must be started within 30 minutes.

1. The user is prompted to uncap and prepare the *QuARTS* reagents near the end of the Methylation & Mutation run. The STARlet unloads the reagent carrier.

The *QuARTS* reagents must be reloaded and the prompt addressed within 60 minutes, or the run will abort.

2. Remove the capped reagents from the carrier, vortex all except TUBES, 200152 to mix, and then spin all, except ELU BFR, 200130, briefly in a centrifuge.

Part #	Component
200130	ELU BFR, Elution Buffer
200131	MIX A, Oligo Mix A, Methylation
200132	MIX B, Oligo Mix B, Mutation
200133	ENZ, Enzyme Mix
200134	D CAL 1, DNA Calibrator 1, High Methylation
200135	D CAL 2, DNA Calibrator 2, Low Methylation
200136	D CAL 3, DNA Calibrator 3, High Mutation
200137	D CAL 4, DNA Calibrator 4, Low Mutation
200152	2 × TUBES, Barcoded Mixing Tubes

3. Remove caps and replace each tube on the carrier in its original location. Select 'Next' to reload the carrier.

Failure to remove cap will abort the run and result in run failure.

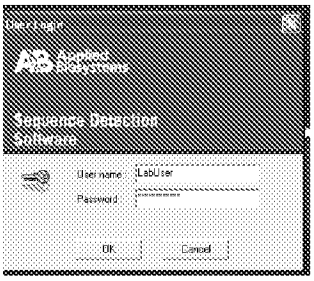
4. As the carrier is reloaded, all reagents in the carrier are scanned and checked against the barcodes scanned at the start of the run. If the barcodes do not match, the carrier is unloaded and the user prompted to correct the reagent placement.
5. At the end of the run, the user is prompted to remove the 96-well *QuARTS* assay plate. Select 'Next' to unload carriers.
6. Seal the plate with adhesive seal (Life Technologies, 4311971).
7. Centrifuge the sealed plate at 1900 × g to 2000 × g for 1 minute.

Run the QuARTS Plate

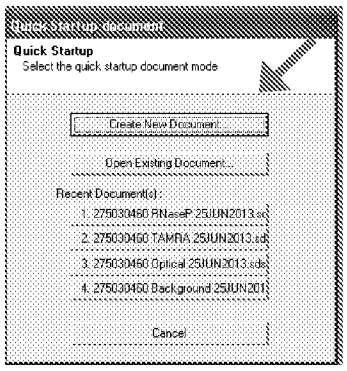
NOTE: Preparation of the 7500 Fast Dx may be completed during the QuARTS* Plate Setup *steps.

1. Place plate into the 7500 Fast Dx.
2. Power on the instrument and computer and log into the 7500 Fast Dx Real-Time PCR

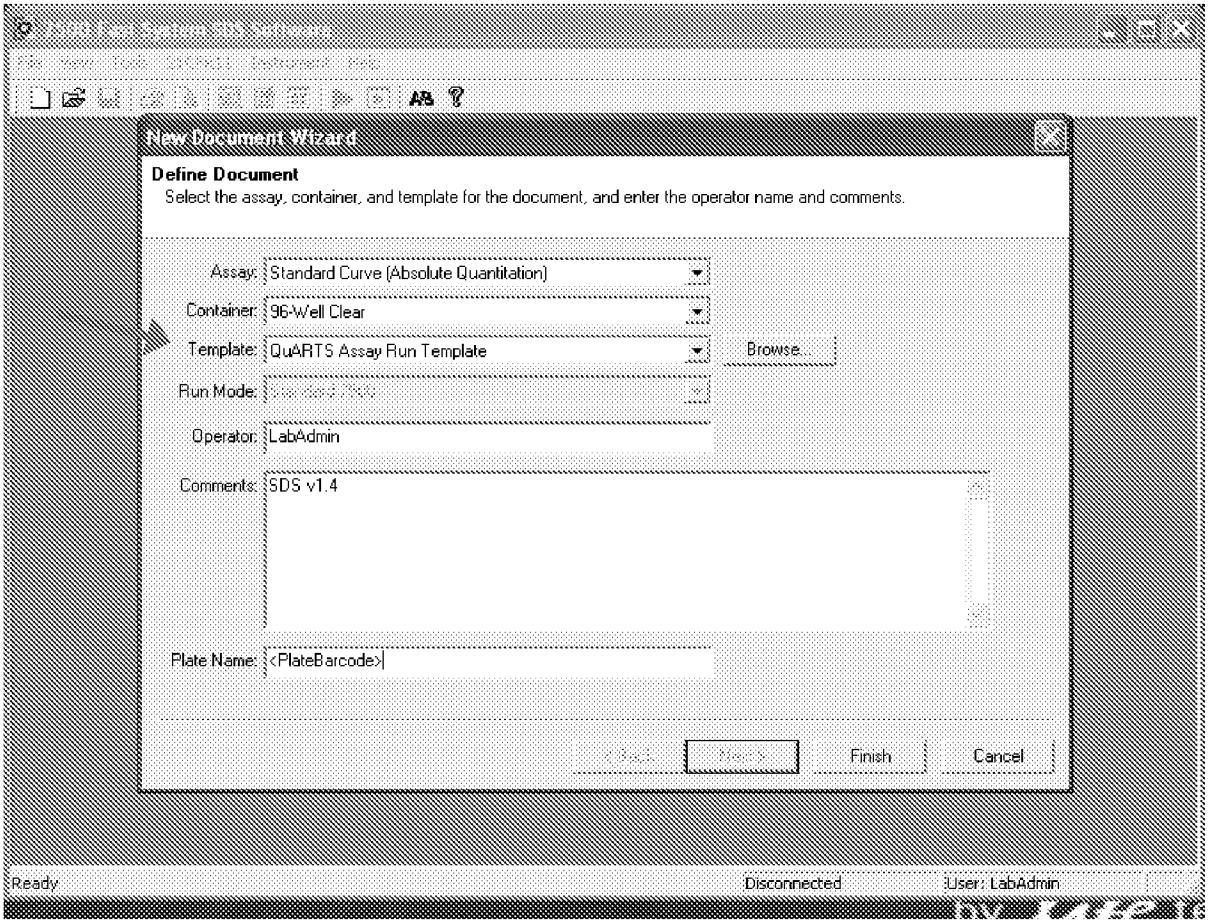
instrument software.



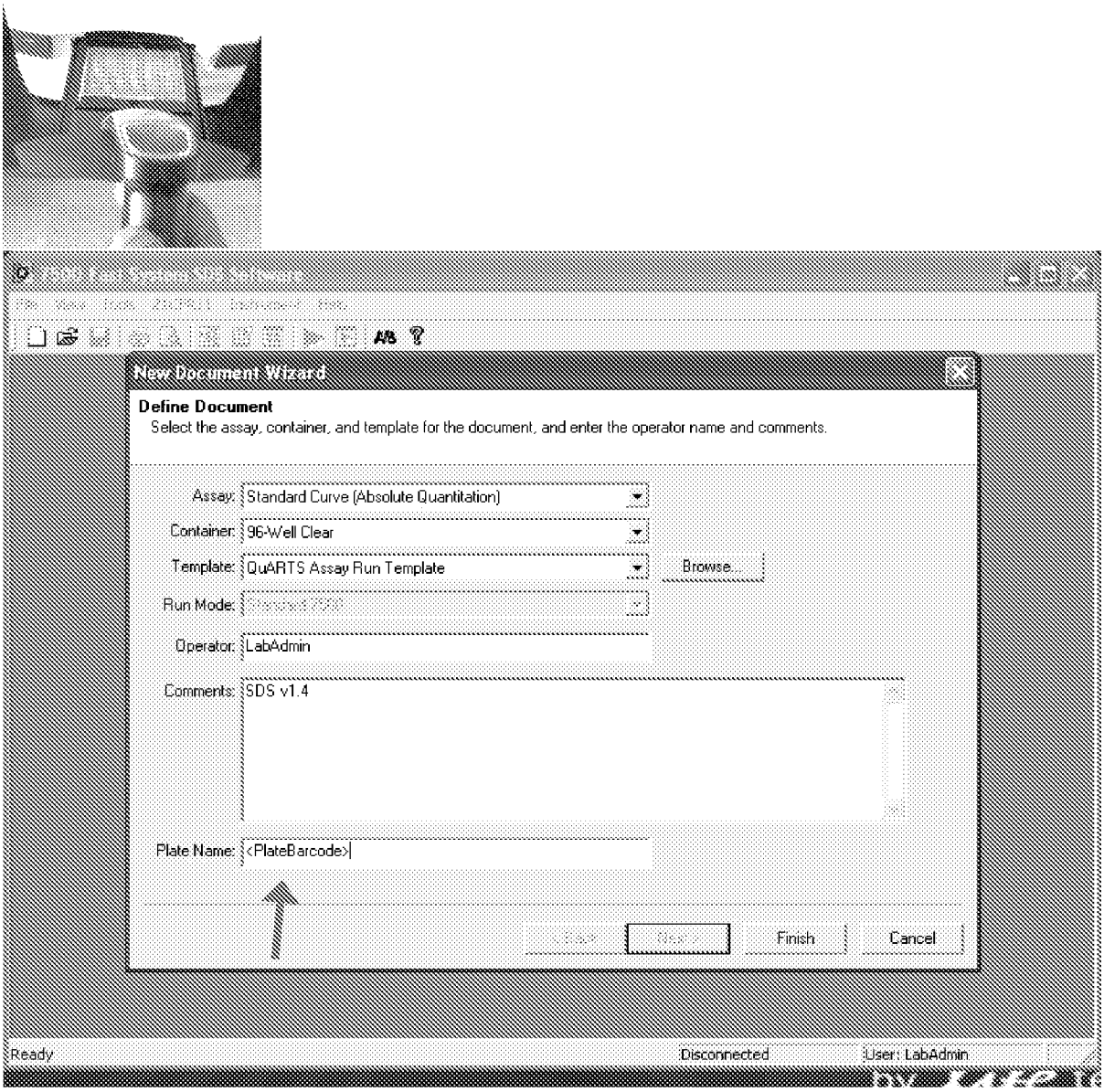
3. Select ‘Create a new document’.



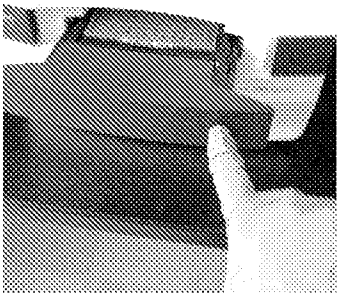
4. Load the Template ‘*QuARTS* Assay Run Template’.



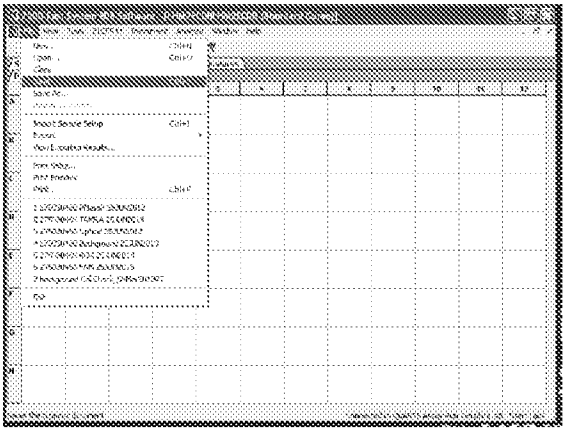
5. Scan the barcode on the plate into the filename as the “Plate Name”.



6. Close the 7500 Fast Dx drawer.

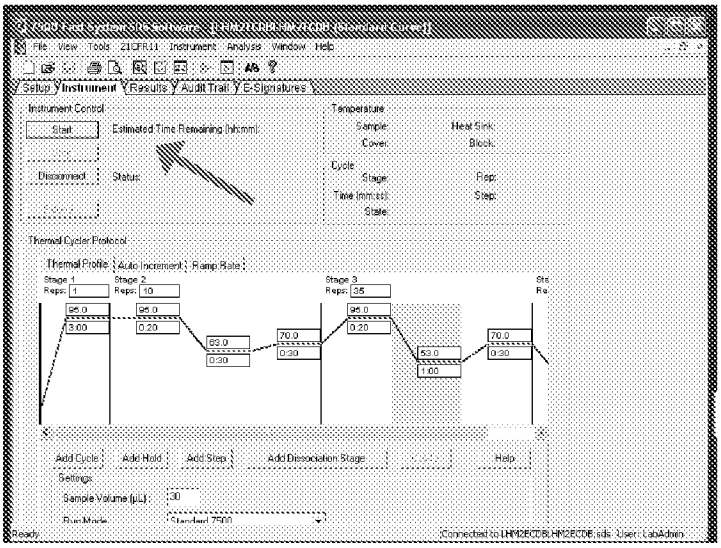


7. After selecting finish, save the run.

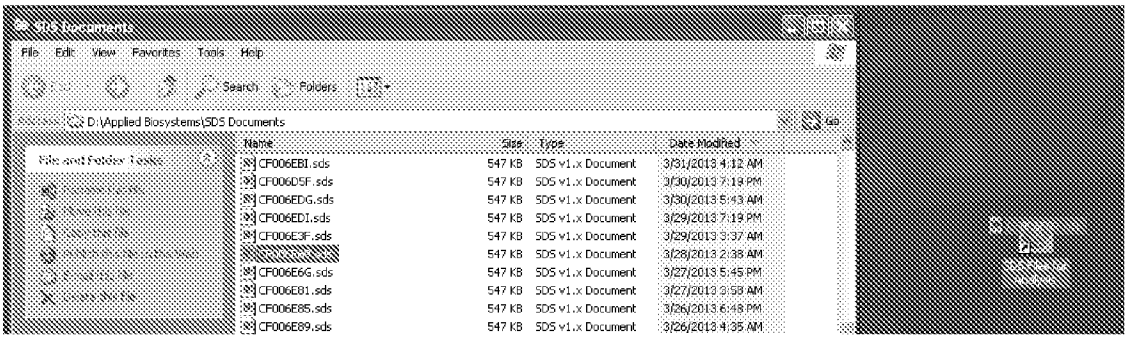


File name must match the plate barcode exactly in order to link assay runs for data analysis.

8. Place plate into the 7500 Fast Dx.
9. Start the run.



10. When the run is complete, using Windows Explorer, open the desktop shortcut labeled "Instrument SDS Files."
11. Copy the <barcode>.sds file, open the Desktop shortcut labeled "SDS Files for Analysis," and paste it into that folder. Alternatively, copy the file to a USB drive for manual transfer to the Exact Sciences System computer running the Analysis Software application.



Hemoglobin Assay

The Hemoglobin Assay plate is set up on the STARlet to ensure tracking of sample positions in a 96-well plate. The remaining assay steps are completed manually. Hemoglobin Assay Supplemental Lot Information (Exact Sciences, 200219) is used with Exact Sciences System Software to enter calibration and lot information for the Hemoglobin Assay. The assay also requires the hemoglobin controls from the *Cologuard* Hemoglobin Control Kit (Exact Sciences, 100073) and entry of the *Cologuard* Hemoglobin Control Kit Supplemental Lot Information (Exact Sciences, 200313).

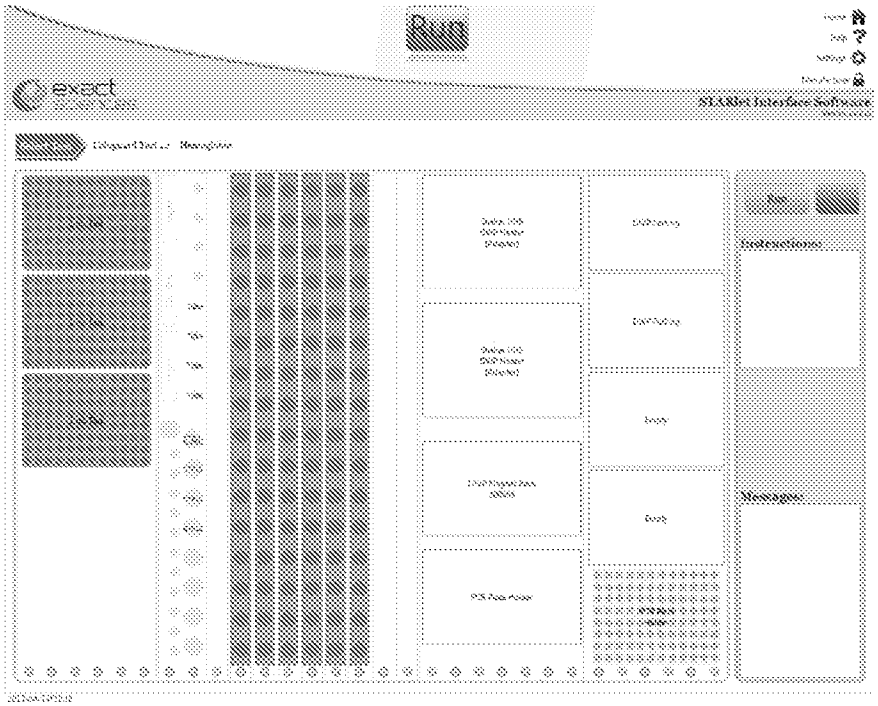
Preparation of Hemoglobin Samples and Reagents

Prepare Samples

1. Remove the hemoglobin samples from storage.
2. Bring hemoglobin samples to room temperature prior to use. From 2 to 8°C storage, let stand 60 minutes on bench top. Tubes may remain at room temperature before sampling for up to 3 hours.

Prepare Hemoglobin Assay Reagents and STARlet

1. Remove Hemoglobin Assay Reagents, including Hb Calibrator (Exact Sciences, 100031) and Hemoglobin controls from *Cologuard* Hemoglobin Control Kit (100073), from storage. Allow to equilibrate at room temperature for 60 minutes.
2. Log into the STARlet Interface Software.
3. Perform daily or weekly maintenance on the STARlet, if required. Refer to *Automated DNA Preparation and QuARTS Assay Setup STARlet Setup* for further instruction.
4. Select the *Cologuard* test and Hemoglobin setup run and select 'Load Setup'.
5. Select 'Run' to start the loading process. The Hemoglobin Assay deck layout appears on the screen.



6. Thirty minutes after equilibration begins, reconstitute the Hemoglobin Assay Calibrator (Exact Sciences, 200146) and Hemoglobin Assay Controls 1-3 (Exact Sciences 100073) each with 1.5 mL deionized or higher grade water.
7. Replace stoppers and invert to ensure any material on the rubber stopper is fully reconstituted.
8. Vortex to reconstitute Hemoglobin Assay Calibrator and Hemoglobin Assay Controls 1-3 (Exact Sciences, 100073) at highest setting for 10 seconds.
9. Continue to equilibrate to the end of the 60-minute period.
10. Inspect Hemoglobin Assay Wash Concentrate (Exact Sciences, 200145) for precipitate. If precipitation is present, warm at 35-50°C for 20 minutes or until solubilized. Invert to mix as needed.
11. Prepare Hemoglobin Assay Wash by performing a 10-fold dilution of the Hemoglobin Assay Wash Concentrate (Exact Sciences 200145). For each plate processed, combine 50 mL of Hemoglobin Assay Wash Concentrate with 450 mL of deionized or higher grade water.

Ensure Hemoglobin Assay Wash is prepared fresh the day of use.

STARlet Setup for Hemoglobin Assay

1. Confirm that Tube sample grooves are void of stool sample. If stool sample remains, vortex at highest speed until grooves of the probe are void of stool sample.
2. Place the Tubes with foil side up into the carriers working back to front, left to right. Orient the tubes so that the barcodes are facing the barcode reader.

Push the tubes completely into the rack positions. Verify that all tubes are in alignment and make adjustments, if needed.

Empty positions are not permitted, except after the last sample tube. Always load from left to right. Load all six carriers regardless of the number of samples, placing empty carriers to the right of loaded carriers.

Carriers with unread sample barcodes will be unloaded. The barcodes must be adjusted and the carrier reloaded until the barcode is successfully rescanned, or barcode sample IDs may be entered manually by the operator.

3. Load Sample Buffer (Exact Sciences, 200143).
   a. Transfer the peel-off barcode from the buffer bottle to a clean 50 mL trough, mix by inversion and transfer all contents to the trough.
   b. Place trough in the indicated carrier position.
4. Load three trays 1000 µL CORE (Hamilton, 235905) pipette tips into the left tip carrier.

Load only full trays of tips, or an invalid run may result.

5. Load 4 uncapped mixing tubes (Exact Sciences, 200152) for calibrator dilution into the appropriate positions on the deck.
6. Vortex Hb CAL vial and Hb CTRL 1-3 vials at highest setting for 10 seconds, remove caps, and place vials in the appropriate position on the Hemoglobin reagent carrier.
7. Using a clean swab for each vial, wipe the inside of the neck of the Hb CAL vial and Hb CTRL 1-3 vials to remove any residual liquid from the vial neck.

Residual liquid inside the neck of the Hb CAL can Hb CTRL vials can interfere with liquid detection and may result in an invalid run.

Wash Hemoglobin Assay Plate

When adding reagents to the hemoglobin plate, add to each column using an 8-channel pipette. Maintain the same order of addition for all subsequent reagent additions.

Automated Hemoglobin Plate Setup procedure must be started within 10 minutes after wash steps are completed.

1. After the end of the 60-minute equilibration period, immediately before automated hemoglobin plate setup, wash the Hemoglobin Assay Plate (Exact Sciences, 200142) five times using the prepared Hemoglobin Assay Wash.
   a. Add 250 µL of prepared Hemoglobin Assay Wash to each well using an 8-channel pipette.
   b. Quickly flip the plate to remove the contents of the plate.
   c. Repeat Steps 1a and 1b four more times for a total of five wash steps.
   d. After the fifth wash, remove residual wash by inverting and tapping on dry paper towels.
2. Inspect the plate to ensure that residual assay wash buffer has been removed.

Residual assay wash buffer could adversely affect assay performance. If residual buffer is present, tap plate upside down on paper towels until removed.

3. Load the washed Hemoglobin Assay Plate into the appropriate carrier and select 'Next'. Follow the instructions on the screen.
4. The instrument reads the plate barcode to ensure validity of the expiration date and that the

lot matches a scanned SLIB.

Automated Hemoglobin Plate Setup

1. Once all required materials are loaded and checks are completed, plate setup begins with the first pickup of tips.
2. When plate setup run is complete, immediately proceed with processing the plate following the *Hemoglobin Assay Procedure*.

Hemoglobin Assay Procedure

1. Remove the assay plate, cover with Sigma Titer Top (Sigma, T-TOPS-100), and incubate at room temperature for 60 minutes.

NOTE: Incubation time from end of Automated Hemoglobin Plate Setup to Step 2 below must be 60 minutes ±5 minutes.

2. Remove cover and invert plate quickly to complete remove the contents.
3. Wash the plate five times.
   a. Add 250 µL of prepared Hemoglobin Assay Wash to each well using an 8-channel pipette.
   b. Quickly flip the plate to remove the contents of the plate.
   c. Repeat Steps 3a and 3b four more times for a total of five wash steps.
   d. After the fifth wash, remove residual wash by inverting and tapping on dry paper towels.

Residual assay wash buffer could adversely affect assay performance. If residual buffer is present, tap plate upside down on paper towels until removed.

4. Add 100 µL of Antibody Conjugate (Exact Sciences, 200144) to each well using the 8 channel pipette.
5. Cover with Sigma Titer Top and incubate at room temperature for 1 hour ± 5 minutes.
6. Remove cover and invert plate quickly to complete remove the contents.
7. Wash the plate five times.
   a. Add 250 µL per well of prepared Hemoglobin Assay Wash using an 8 channel pipette.
   b. Quickly flip the plate to remove the contents of the plate.
   c. Repeat Steps 7a and 7b four more times for a total of five wash steps.
   d. After the fifth wash, remove residual wash by inverting and tapping on dry paper towels.

Residual assay wash buffer could adversely affect assay performance. If residual buffer is present, tap plate upside down on paper towels until removed.

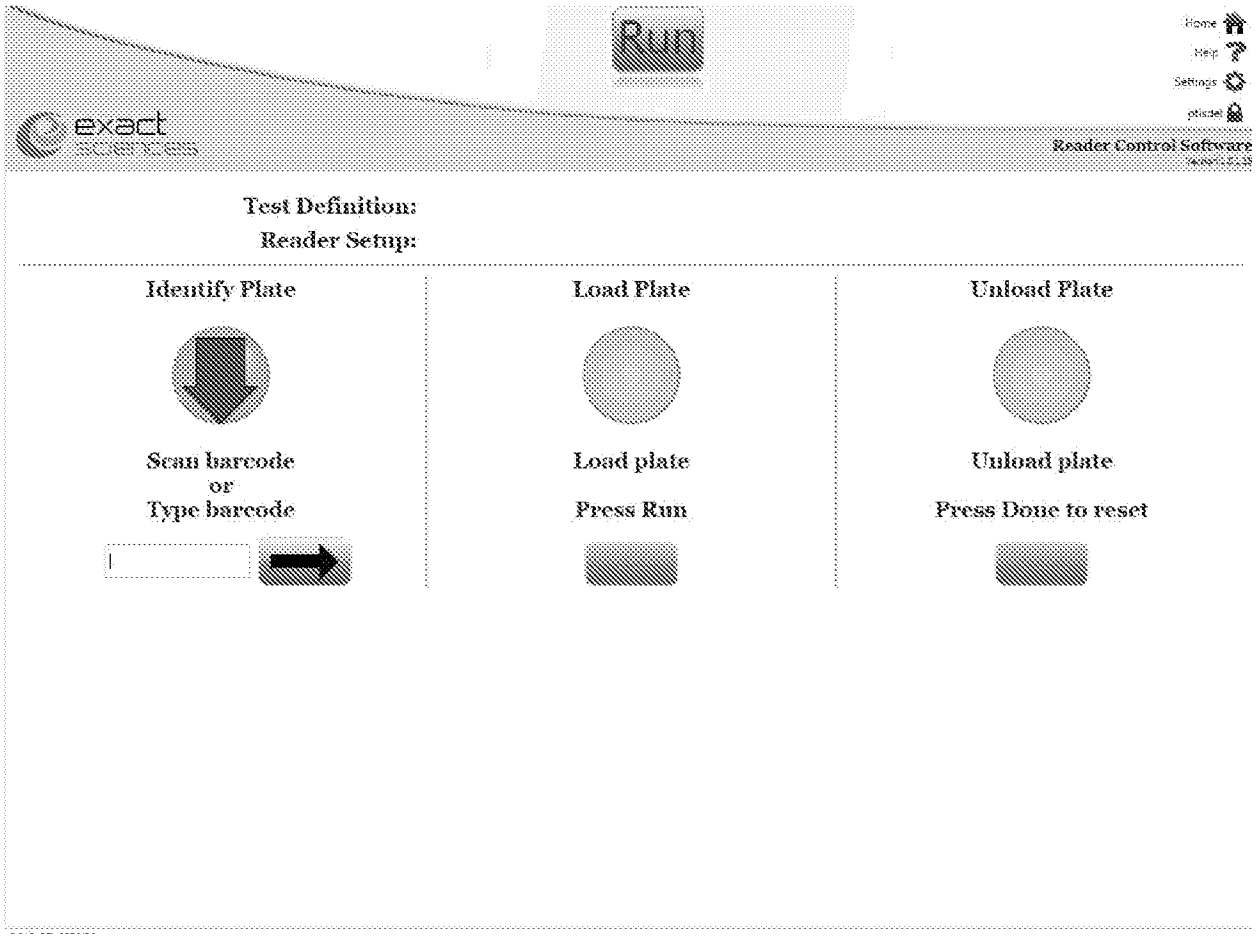
8. Add 100 µL Substrate (Exact Sciences, 200100) to each well of the plate using an 8-channel pipette.
9. Cover with Sigma Titer Top and incubate at room temperature for 15 minutes ± 1.5 minutes.
10. Remove cover and add 100 µL of Stop Solution (Exact Sciences, 200101) to each well.
11. Proceed immediately to Step 1 of *Read Hemoglobin Plate* below.

Read the Hemoglobin Assay Plate within 15 minutes of addition of the Stop Solution.

Read Hemoglobin Plate

NOTE: The Reader Control Software may be used during a STARlet run. If needed, leave the Exact Sciences STARlet Interface Software logged in and running and switch to the Reader Control Software.

1. Log into the Exact Sciences Reader Control Software. The Run screen appears:



2. Scan or enter the barcode of the Hemoglobin plate. For manual entry of barcode, repeat the barcode entry in the second field if required by site administrator. After typing the barcode(s), select the arrow button to continue.
3. Load plate into the BioTek reader when prompted by the software.

NOTE: Place the plate into the reader so that embossed "A1" is positioned in the back left corner of the reader tray. Incorrect placement may result in invalid or incorrect results.

4. Press 'Run' to read the plate.
5. Unload the plate when prompted to do so.
6. Press 'Done' to reset the software to be ready to read a new plate.
7. The assay data are automatically transmitted to the Analysis Software.

8. In the event that a connection to the Exact Sciences Analysis Software is not available, data may be transferred manually. Copy the data file from C:\ExactSciences\Reader\Runs with file name <plate barcode>.<checksum>.reader to a USB drive if data needs to be transferred between computers.

Hemoglobin Sample Storage

1. When the hemoglobin plate setup steps are completed, cover the used Tubes with water-resistant wrap (e.g., Parafilm or plastic wrap) and store with foil side up at 2 to 8°C for up to 7 days or freeze at <-15 °C for longer storage.
2. If repeat testing is required, see *Retest Hemoglobin Sample Tubes* in *Procedural Notes and Precautions* for detailed instructions.

Data Handling and Analysis

Data from the Methylation, Mutation, and Hemoglobin Assays are integrated and analyzed by the Exact Sciences Analysis Software. The Exact Sciences Analysis Software maintains traceability of the sample to result through sample barcodes scanned during the hemoglobin and molecular (methylation and mutation assays collectively) assay plate setup runs. For the molecular assay, data from the thermocycler (7500 Fast Dx) are imported into the Exact Sciences Analysis Software and the fluorescent signal for each channel versus cycle time is analyzed to calculate a crossing point (Cp) where the detection threshold is exceeded. This value enables the calculation of detected concentration of each DNA marker using the respective calibrators. For the Hemoglobin Assay, the optical density data is imported from the reader and the hemoglobin concentration in each sample and control is calculated from respective calibrators.

The system uses the expected values and actual results of the calibrator and control samples to assign a run status (valid/invalid) for Methylation, Mutation and Hemoglobin Assay runs. Users review, comment upon, or invalidate sample or run data in the software as required to capture any errors or invalid samples that occur during the assay procedure.

The software calculates an overall *Cologuard* score for each sample by combining the released results of each marker for that sample (linked by sample ID). A Negative or Positive result is assigned for each sample based on the *Cologuard* score. Invalid *Cologuard* results occur if any constituent assay results are invalid. Details on the use of the software can be found in the Exact Sciences System Software User's Manual.

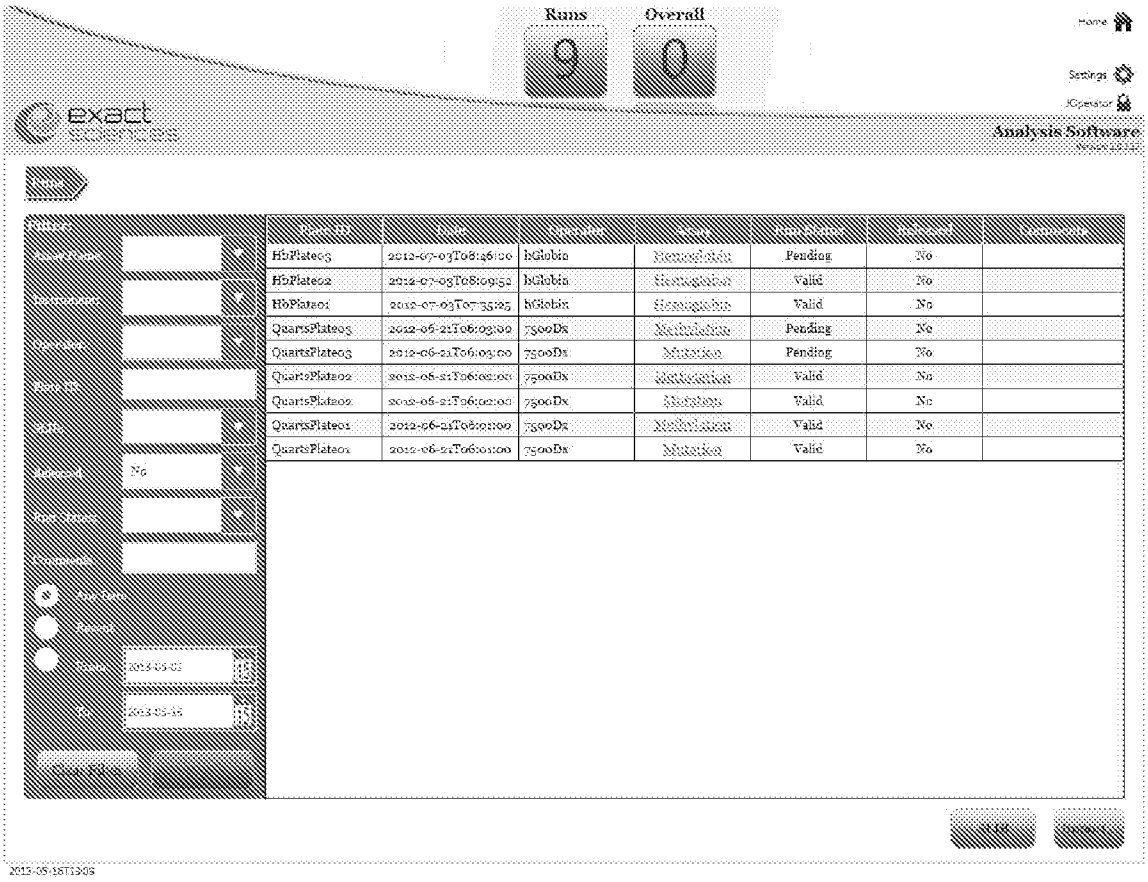
The barcoded identification numbers on the hemoglobin sample and the DNA sample must match for Hemoglobin and DNA assay results to be matched into an overall *Cologuard* result. If a different identification number is assigned to the DNA sample, the same identification number must also be assigned to the corresponding Tube.

The barcodes affixed to the hemoglobin sample and the DNA sample must follow the appropriate barcode format, resolution, placement and ANSI/ISO specifications as directed by the STARlet instrument manual.

Review and Release Methylation, Mutation, or Hemoglobin Assay Results

Once the assay runs are complete, the run data are imported into the Analysis Software and the assay run and individual sample assay results are calculated. Results of each assay are reviewed before the software incorporates the results into the calculations that generate the *Cologuard* result. Users must release assay results in the order that they are run.

1. Log into the Analysis Software.
2. The Runs screen displays a list of recent runs that have not been released. Apply filters to locate the run to be released in the Runs table.



NOTE: To navigate back to the Runs screen at any time, select the 'Runs' button on the top of the screen. Filters will need to be re-applied.

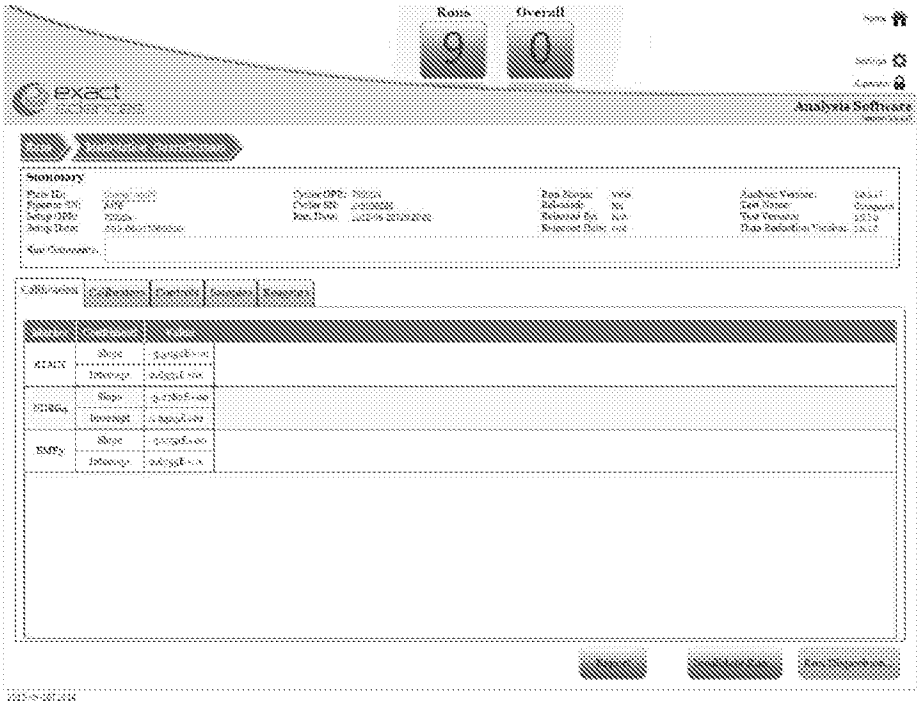
3. If run data files need to be manually imported (e.g., the computers running the other Exact Sciences System Software applications (STARlet Interface and Reader Control) are not connected to the computer running the Analysis Software), follow these steps:
   a. Select 'Import' on the bottom right of the screen to select a file to upload.
   b. Connect the USB drive containing the data and select the file to upload. Valid file types from STARlet Interface have a .plate extension, and files from the Reader Control have .reader extensions.

NOTE: Altering file names may make files invalid for import.

4. If the 7500 Fast Dx Real-Time PCR Instrument computer is not networked to the computer running the Analysis Software, copy the SDS file from the ABI computer. (SDS files are typically saved in the "Instrument SDS Files" folder on the desktop or the D:\Applied Biosystems\SDS Documents\ folder). Save the SDS file on an USB drive, transfer memory stick to Analysis computer, and upload file to Analysis Software using the 'Import' button.
5. Once the run list includes the desired run, select the hyperlink under the Assay column to display Run details.

Run ID	Date	Operator	Assay	Run Status	Archived	Comments
QuartsPlate01	2012-06-21T06:01:00	7500DX	Mutation	Valid	No	
QuartsPlate02	2012-06-21T06:02:00	7500DX	Methylation	Valid	No	
QuartsPlate02	2012-06-21T06:02:00	7500DX	Mutation	Valid	No	
HbPlate01	2012-07-03T07:35:25	hGlobin	Hemoglobin	Valid	No	

6. The run detail screen displays run information in the Summary section and on tabs for Calibration, Calibrator, Controls, Samples, and Reagents.



7. Review data as needed in Calibrator, Calibration, Controls, Samples, and Reagents tabs.
   a. Each tab has specific information about the results of the testing for the displayed run. Select each tab to review the data.
   b. Plate IDs match the barcode on the 96-well plate used in the setup run.
   c. For the Hemoglobin setup run, one Plate ID is linked to one "Hemoglobin" assay run in the Assay column.
   d. For the Methylation & Mutation setup run, one Plate ID is linked to one "Methylation" assay and one "Mutation" assay in different rows of the Assay column.
   e. The run status of the assays on a plate is marked as Pending until assay run data for the plate are uploaded from the 7500 Fast Dx Real-Time PCR Instrument SDS file or the Reader Control Software.

f. When an assay run status is either Valid or Invalid, the assay run results are ready for user review.

8. If user comment is required, follow these steps.
   a. If user comment is needed for an individual control, calibrator, or sample, select the 'Comment' area for the sample. Enter the comment and select an area outside the comment area to save the comment.
   b. To comment on an entire run, select the 'Run Comments' field in the Summary section and enter the comment.
   c. Each calibrator, control, and sample has a status listed as Valid or Invalid.
9. In the event of errors in manual processing steps or other errors observed, a user may invalidate sample results.

NOTE: Ensure that invalidation of individual sample results is completed prior to releasing the run.

   a. To mark individual sample assay results as invalid, go to the Samples tab, select the checkbox for the individual sample (s), and select the 'Invalidate Samples…' button. Enter comment, user name and password to complete the action.

Sample results invalidated by a user are permanently marked as invalid for that particular run once Run Disposition is performed and run data are released or closed. Invalidation/Undo Invalidation cannot be performed on released or closed sample results

   b. To mark all the results in a run invalid, select the 'Invalidate Run…' button. Press yes to confirm the invalidate action and enter Username and Password to complete the action.

Runs invalidated by a user cannot be released through a Run Disposition. Invalid assay results may only be Closed.

10. To undo user invalidation, select the checkbox for the individual sample and select on the 'Undo Invalidate…' button. Enter Username and Password to complete the action.
11. To release a run of assay results, select the 'Run Disposition' button on the bottom of the screen and enter Username and password to complete the action.

Invalid or Pending runs cannot be released, they may only be dispositioned as Closed.

Results from Closed runs are not available for overall test result interpretation.

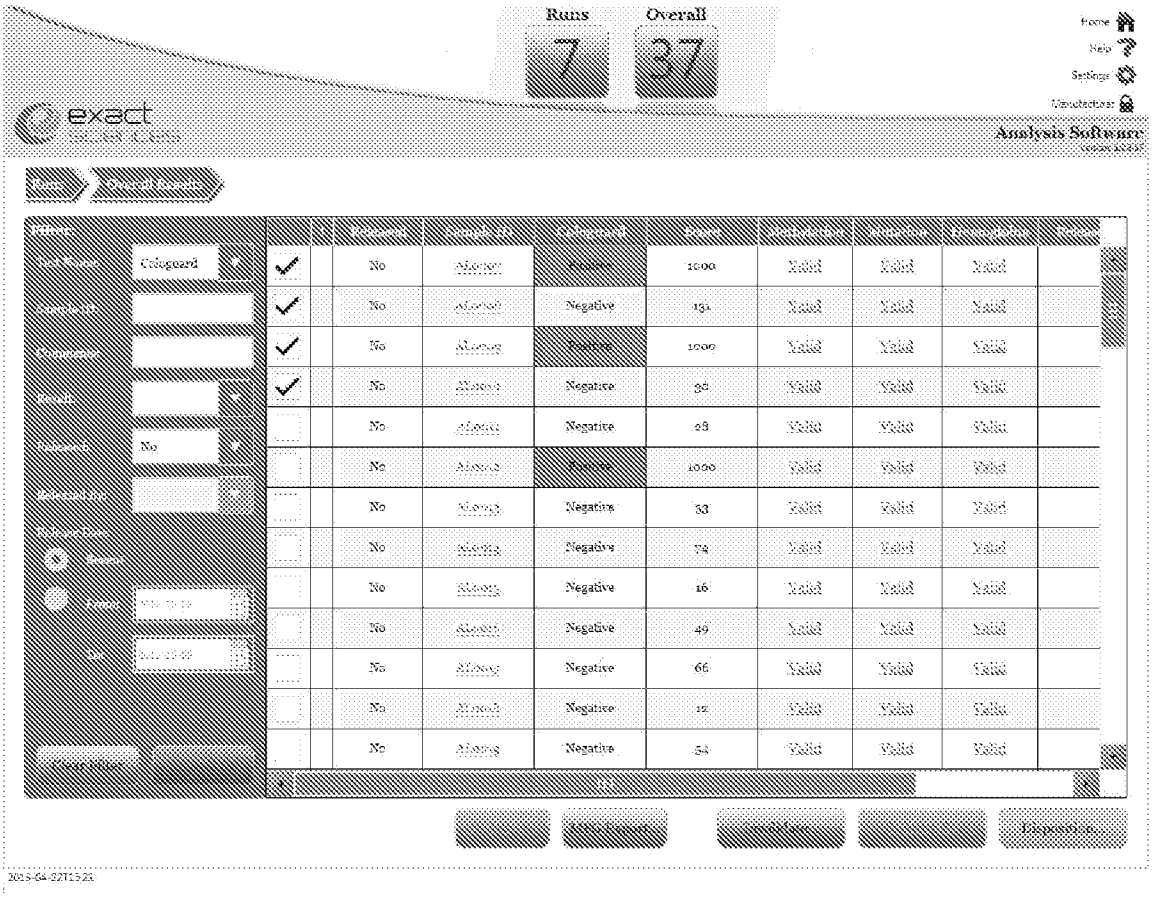
Runs that have been released or closed cannot be invalidated by any user, nor can invalidation be undone on a released or closed run.

12. Releasing an assay run makes the results available for calculation of the overall *Cologuard* test result for the samples in the run.
13. To generate an assay run report, select the 'Report' button on the bottom of the screen. The report may be printed or saved to a PDF file.

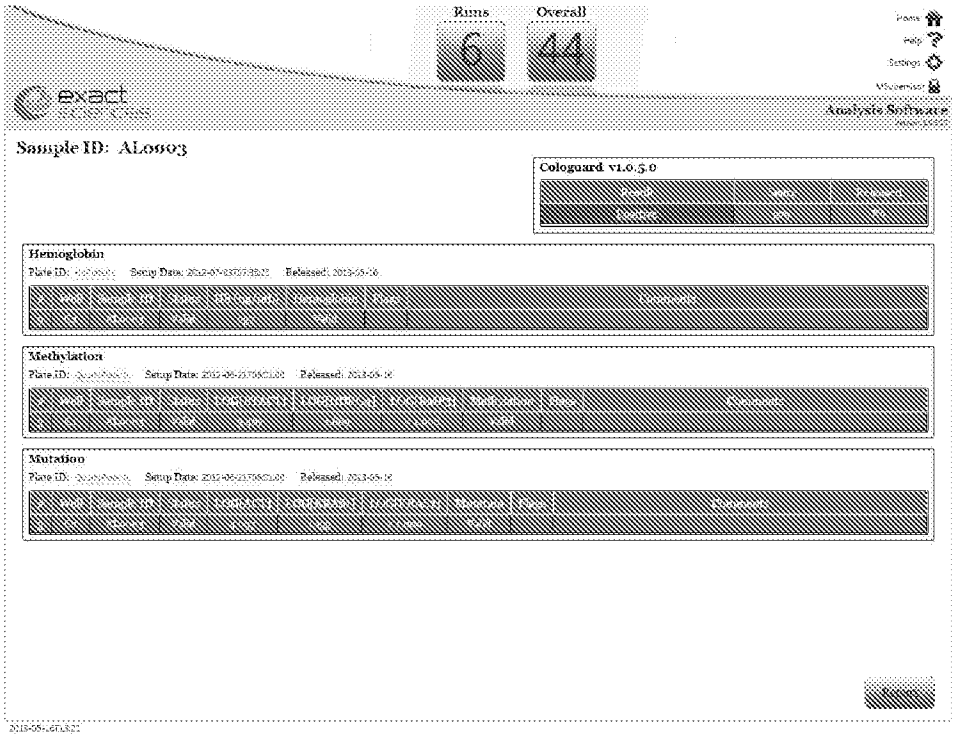
Review and Release Overall *Cologuard* Results

After users confirm and release valid Methylation, Mutation, and Hemoglobin run results, the software generates an overall *Cologuard* score for each sample using each of the marker results for that sample. A Negative or Positive result is assigned based on the *Cologuard* score. Invalid *Cologuard* results occur if any of the constituent assay results are invalid. Active users in the Supervisor or Administrator role may disposition (release or close) overall *Cologuard* test results. Once matched samples from Methylation, Mutation, and Hemoglobin Assay runs have been released, overall test results for each sample are available.

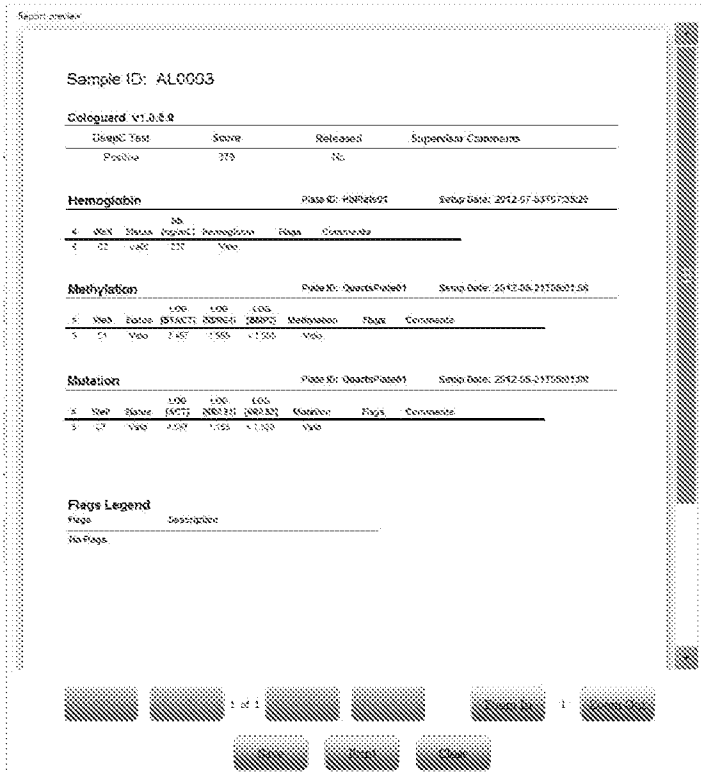
1. Log into the Exact Sciences Analysis Software.
2. Select the 'Overall' button to view the overall test results table. The filter defaults to show results that have not yet been released. Filters may be applied to narrow the table to show only specific samples or types of results.



   a. For each sample, ! (Flags), Released, Sample ID, Test Result, Score, and Methylation, Mutation, and Hemoglobin run status are displayed on the Overall screen.
   b. To select a group of samples for action, enter a checkmark in the samples selection box.
3. To review a summary of individual assay results for a sample, select the hyperlink for the Sample ID.
   a. The Sample Detail report with overall result and individual assay results for the selected sample are displayed.



b. The Report button displays a printable copy of the sample detail report for the selected rows that can also be saved to PDF file.



4. In the event of errors in manual processing steps or other errors observed, a Supervisor-level user may invalidate overall test results before they are dispositioned.

a. To invalidate an overall test result for a sample, go to the Overall screen, select the checkbox next to the sample, and touch the Invalidate button. A confirmation screen appears. Select 'Yes' to confirm and enter your comment, username and password to complete the action.

Samples invalidated by a user are permanently marked as invalid once a Disposition is performed and sample results are released.

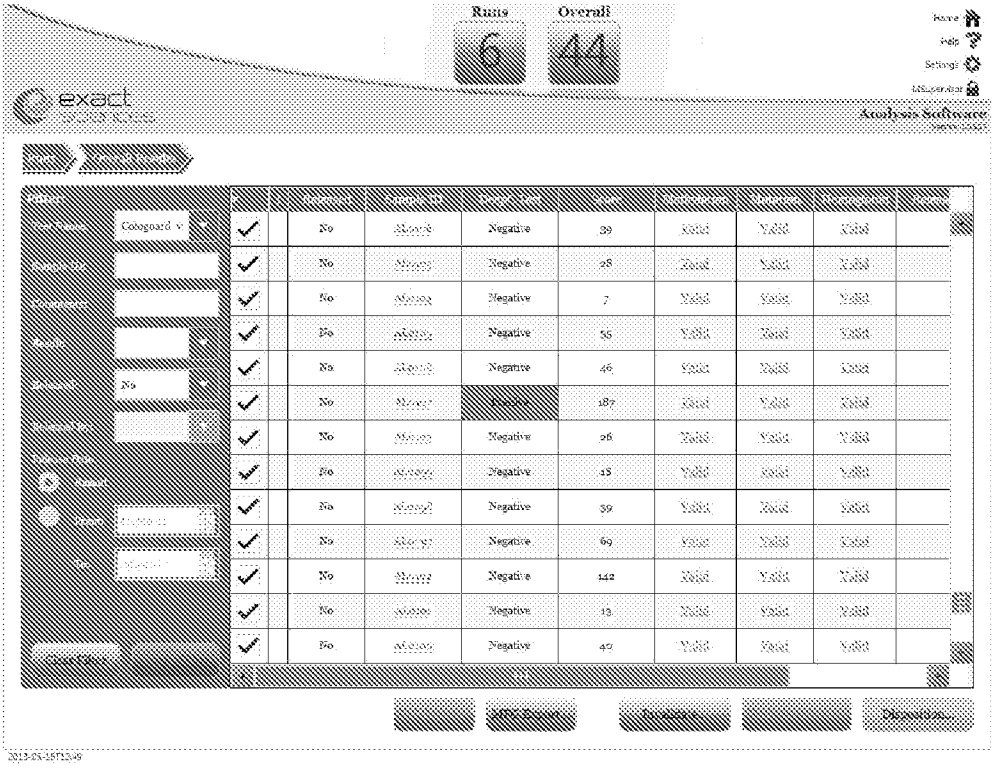
b. To undo user invalidation, select the checkbox for the individual sample and select the 'Undo Invalidate…' button and enter your username and password to complete the action.

5. To disposition the overall results for a sample or group of samples, select the checkbox(s) beside the sample(s) and select the 'Disposition' Button on the bottom of the screen. Enter Supervisor-level username and password, then:

NOTE: The column header can be used to select all the samples displayed in the Overall results table in order to release or close multiple results simultaneously. The user must double-click on the column heading.

NOTE: If all selected results cannot be released, the 'Release' button will not be available.

a. Select 'Release' to release overall test results for export. Released overall test results are automatically written to LIS Export file. Released overall test results are written to MFG Export files when the MFG Export function is selected.
b. Alternatively, select 'Close' to indicate that the results should not be exported. Closed results cannot be exported in LIS Export or MFG Export files.



6. Select the 'Disposition' button and enter user credentials to release or close the selected sample results. If a button is not enabled, the corresponding action is not available for all of the selected samples.

Interpretation of Results

The Exact Sciences System Software imports run data into the Analysis Software. The software calculates assay results for controls and individual samples. Results of each assay, methylation, mutation, and hemoglobin, are reviewed prior to the release of the assay run data in the software. Prior to release assay run results, deviations to process in the assay set up are noted for affected samples. If a deviation (e.g., operator error, instrument error) occurs, the deviation may compromise the results of the test regardless of the control validity. In the event that a sample result is compromised, the individual result may be invalidated. After results are reviewed for deviations, results may be released for the entire assay run. If an assay control fails, or the operator invalidates an entire assay run, no sample results will be present for that assay and all samples in the run must be retested. Only valid sample results from valid assay runs are used to calculate an overall *Cologuard* score.

Users may also review overall *Cologuard* results and invalidate sample results as needed based on events and issues known to the user, such as individual sample or reagent contamination, process errors, or automation abort with unconfirmed completed steps. Users may enter comments in the software for any sample result that is invalidated by the user for a known technical error that cannot be detected by the software.

As users confirm and release valid methylation, mutation, and hemoglobin results for an assay run, the Analysis Software will link the constituent assay results by sample ID and calculate a *Cologuard* score. The score is used to assign the final *Cologuard* result: Positive, Negative, or Invalid. Valid *Cologuard* results may be released and reported. An invalid *Cologuard* result occurs if the sample result from any constituent assay is invalid.

Procedural Notes and Precautions

Additional Stool Homogenate Aliquots

If additional aliquots of stool homogenate are desired for testing or for sample archive, label and prepare additional tubes as in *Preparation of Stool Homogenate* and store as directed.

1. Do not store samples in a Stool Container. Any additional homogenate aliquots should be processed and stored in 50 mL tubes at the same time as testing aliquots.
2. Homogenate aliquot tubes can be stored frozen (< -15°C) for 1 month.
3. Homogenate aliquot tubes can be stored frozen (<-70°C) for 2 years.

Insufficient Supernatant

If less than 5 mL of supernatant are obtained from a sample during *Prepare Samples and Perform DNA Capture, Prepare Supernatant* and additional stool sample aliquot tubes are available, the prepared supernatant may be stored until additional material has been obtained, as described in Steps 5b and 15b of the *Prepare Supernatant* section. If 5 mL of supernatant are still not obtained or additional sample aliquot tubes are not available, discard supernatant and request a new sample.

Enter Supplementary Lot Information

Use the following procedures to support STARlet Setup when required.

1. Log in to the Analysis Software.

2. Scan all of the 2D barcodes on the SLIB sheet in any order. Each scan is acknowledged in a pop-up dialog. When all barcodes in a SLIB have been scanned, the entry status is indicated as "added" or "already been imported".
3. Supplementary lot information loaded into the system can be viewed in the SLIB summary table by selecting the SLIB button in the Analysis Software.

Prepare Capture Beads (for >6 tubes)

The "Prep Beads" protocol referred to in Step 9 of *DNA Capture, Prepare Capture Beads* aspirates up to six tubes of beads. If operator prefers to prepare greater than six tubes, use the following steps.

1. Set the Capture Incubator (Exact Sciences, 300546) to preheat ("Bead Prep 1" program).
2. Allow Capture Beads (Exact Sciences, 200150) to sit at room temperature for a minimum of 30 minutes.
3. Vortex Capture Beads at highest setting for 30 seconds to suspend the beads.
4. Label the 50 mL conical tubes with Capture Bead preparation date and lot information.

Labels used in the Capture Incubator have specific requirements for size, material and thickness. Label the bead preparation tubes using permanent marker or refer to Capture Incubator User's Manual for detailed label specifications.

5. Transfer 3.25 mL of beads to each of the labeled 50 mL tubes.
6. Add 10 mL of Capture Bead Pre-wash (Exact Sciences, 200120) to each tube and secure the 50 mL tube cap.
7. When the Capture Incubator has reached temperature and display prompts user to insert test tubes, place tubes in the Capture Incubator. Close cover and press the 'Start/Select' button to proceed with the cycle.
8. When the cycle is complete, remove tubes from the incubator and place in the centrifuge with appropriate balance tube, if necessary. Centrifuge the tubes until the centrifuge reaches 500 × g and hold for 1-10 seconds.
9. Remove caps and transfer the tubes to the Capture Aspirator (Exact Sciences, 300490). Execute the "**Bind 10 min**" protocol to remove supernatant from the tubes.
10. When aspiration run is complete, remove tubes from the Capture Aspirator and add 3.25 mL of fresh Capture Bead Pre-wash solution to each tube, replace caps, and vortex at highest setting until all Capture Beads are suspended.

NOTE: Once the Capture Beads have been prepared, they can be stored in closed tube for up to 7 days at 2-8°C before use.

Procedure for Performing Partial Runs

Each *Cologuard* kit contains sufficient materials to test 96 samples including 86 patient samples, required controls and calibrators. If fewer than 86 patient samples are processed, use the following guidelines to ensure valid results.

1. DNA Capture steps are performed manually and are processed in sets of up to 22 patient samples. At least one positive DNA Control (D CTRL 1 or D CTRL 2) is required for every distinct set of DNA Capture samples.
2. Input samples for automated processing include a maximum of 43 samples with corresponding controls for a total of 46 samples and controls per batch. In maximum batch

sizes, two full sets of DNA capture samples (46 samples and controls) are used in each batch of automated processing on the STARlet (DNA Preparation and *QuARTS* Assay steps). To process the suggested 86 samples, two batches of DNA Preparation and *QuARTS* Assay steps are to be performed. Reagent fill volumes are designed to be sufficient for this full capacity of two automated batches. Leftover reagents must be discarded at end of run, even if less than a full run of samples was performed.

3. In maximum batch sizes, Hemoglobin Assay steps are performed in 96 well assay plates, using the STARlet for plate setup, followed by a 96 well ELISA based assay. Hemoglobin Controls (Hb CTRL 1, Hb CTRL 2, and Hb CTRL 3) are required for each batch of Hemoglobin Assay samples. Reagent fill volumes are designed for full capacity. Leftover reagents must be discarded at end of run, even if less than a full run of samples was performed.
4. All three DNA controls are required for each DNA plate setup run, regardless of the number of samples run.
5. Use full reagent containers and troughs for setup runs to avoid invalid results. The system does not adjust reagent usage for runs with less than the maximum number of samples.
6. Discard leftover reagents at the end of the run.

Quality Control

Process Controls

1. Required controls must be present in each assay plate setup run to achieve valid results. The system software will not proceed with the method if not all required controls are present.
   a. Input samples for *Automated DNA Preparation and* QuARTS *Assay Setup* must include D CTRL 1, D CTRL 2, and D CTRL 3 in each setup run.
   b. Hemoglobin Controls (Hb CTRL 1, Hb CTRL 2 and Hb CTRL 3) are required for each setup run of Hemoglobin Assay samples.
2. Process controls must yield expected results, or the assay run will be invalid. Allowed ranges for control results are defined by the *Cologuard* DNA Control Kit Supplemental Lot Information (Exact Sciences, 200315) and *Cologuard* Hemoglobin Control Kit Supplemental Lot Information (Exact Sciences, 200313) for each lot of controls.

Lot Matching and Sample Tracking during Processing

1. Users are responsible for ensuring that reagent lots used in manual processing steps are correctly lot matched to reagents used in automated processes. Refer to the DNA and *QuARTS* Reagents Supplemental Lot Information (Exact Sciences, 200218) and the Hemoglobin Assay Supplementary Lot Information (Exact Sciences, 200219).
2. Users are responsible for tracking sample IDs and documentation of processing errors from manual Processing and DNA Capture steps.

Review and Release *Cologuard* Results

1. As users confirm and release valid methylation, mutation, and hemoglobin results for a run, the software will match the results by sample ID and generate an overall *Cologuard* result of Positive, Negative, or Invalid. Sample results invalidated during previous review steps will be called 'Invalid.'
2. Users should review and comment upon overall *Cologuard* results and invalidate sample results as needed based events and issues known to the user.

Troubleshooting Guide

DNA Capture

Centrifuged Stool Sample Abnormalities

1. If the solid/liquid interface of a centrifuged stool sample is unclear, remove 14 mL from the topmost portion of the sample during *Prepare Samples and Perform DNA Capture, Prepare Supernatant*, Step 5.
2. If a solid layer is present above the liquid layer of the centrifuged stool sample, hold the tube at an angle with the tip below the solid layer while pipetting to avoid aspiration of the solid material.

Incomplete Dispersion of Inhibitor Removal Tablet

If Inhibitor Removal Tablet does not immediately disperse during *Prepare Samples and Perform DNA Capture*, *Prepare Supernatant*, Step 5, use the following procedure.

1. Vortex the sample and inhibitor removal tablet at highest setting until tablet breaks apart.

If step above is unsuccessful in breaking up and dispersing the Inhibitor Removal Tablet, request a new sample, as this sample is considered invalid.

2. Proceed with Prepare Samples and Perform DNA Capture, Prepare Supernatant, Step 6.

Broken Spin Filter

Spin filter failure while centrifuging the "SPN" tubes during *Prepare Samples and Perform DNA Capture*, *Prepare Supernatant*, Step 12, is evident by the presence of white inhibitor removal tablet particles present in the filtrate. If this is noted, use the following procedure.

1. Remove the broken spin filter from the SPN tube and cap the tube using a clean cap.
2. Shake the tube to mix sample and the dispersed inhibitor removal tablet and then transfer to a new labeled tube fitted with a spin filter.
3. Place spin filter tube into the centrifuge. Ensure that centrifuge is balanced and spin for 6 min at 3300 × g.
4. Proceed with Prepare Samples and Perform DNA Capture, Prepare Supernatant, Step 13.

Capture Incubator Produces Error Message

If the Capture Incubator produces an error message while running the EXAS8 program during *Prepare Samples and Perform DNA Capture*, *Capture Incubation,* Step 7, refer to the Capture Incubator User's Manual. A description of each error code displayed on the Capture Incubator can be found in the Appendices.

Capture Aspirator Produces Error Message

If the Capture Aspirator produces an error message or a power outage while running the "BIND 10 min" program during *Prepare Samples and Perform DNA Capture*, *Capture Incubation,* Step 9, refer to the Capture Aspirator User's Manual (Troubleshooting section).

Incomplete Aspiration of Supernatant

If liquid remains in the 50 mL conical tubes after aspiration during *Prepare Samples and*

Perform DNA Capture, *Capture Incubation*, Step 8, use the following procedure.

1. Bring tube volume to 10 mL using Capture Wash.
2. Mix to ensure that beads are suspended in liquid.
3. Place tube in Capture Aspirator and repeat the "BIND 10 min" program.

NOTE: Empty positions in rows that contain sample tubes should be occupied to ensure optimal aspiration. Place a 50 mL tube filled with 17.5 mL of water into each empty space.

4. Proceed with Prepare Samples and Perform DNA Capture, Prepare Supernatant, Step 10.

Sample Appears Gelatinous After Aspiration

If a sample appears gelatinous after aspiration during *Prepare Samples and Perform DNA Capture*, *Capture Incubation*, Steps 10-11, observe one of the following recommendations.

1. Re-aspirate sample following Troubleshooting Procedure for Incomplete Aspiration of Supernatant.
2. Continue to process sample if it appears that it will not cause an issue on the automated platform.
3. If sample still appears too gelatinous to run on the automated platform, discard and request a new sample.

Beads Not Fully Suspended After Shaking

If beads remain on the sides of the 50 mL conical tubes after shaking during *Prepare Samples and Perform DNA Capture*, *Capture Incubation*, Step 12, use the following procedure.

1. Rotate tube in Shaker rack, and mix for 1 minute at 400 RPM.
2. Confirm that Capture Beads are suspended in tube.
3. If beads are not suspended, rotate tube and repeat Steps 1 and 2.

DNA Preparation and *QuARTS* Assay

Methylation/Mutation Setup Run Aborts

1. If samples have not been transferred from the 50 mL conical tubes to the deep-well plate, place a new cap on the sample tubes and store at 2 to 8°C until ready to rerun in the methylation/mutation setup.
2. If samples have been transferred to the deep-well plate, discard all samples and reagents on deck. Samples must be repeated from Step 1 of *Prepare and Label Sample Tubes, Prepare Samples and Perform DNA Capture.*

Hemoglobin Assay

Hemoglobin Setup Run Aborts

1. If samples have not been punctured, place at 2 to 8°C until ready to rerun in the Hemoglobin setup. Samples may remain at room temperature for up to three hours. To rerun the setup, begin with *Prepare Hemoglobin Assay Reagents and STARlet*, Step 1.

2. If samples have been punctured, rerun within 3 hours or cover with water-resistant cover and store foil-side up at 2 to 8°C until ready to rerun the Hemoglobin setup. Before rerunning, confirm that all sample tubes are correctly placed into sample racks.

Retest Hemoglobin Sample Tubes

If repeat testing is required due to a Hemoglobin Assay run failure or individual sample failure in a Hemoglobin Assay, the following procedure should be used for repeat testing.

1. When initial hemoglobin plate setup steps are completed, cover hemoglobin sample tubes with a water-resistant cover and store in racks with foil side up at 2 to 8°C for up to 7 days.
2. Remove punctured hemoglobin sample tubes from 2 to 8°C and equilibrate to room temperature.
3. Remove plastic wrap and proceed with Hemoglobin Assay, Preparation of Samples and Reagents, Prepare Samples, Step 4.

Procedural Limitations

- DO NOT mix or substitute reagents from Supplemental Lot Information containing different lot groupings.
- DO NOT use any reagent after its expiration date.
- DO NOT store reagents in “frost-free” freezers.
- Only use with specimens collected with the *Cologuard* Collection Kit (Exact Sciences, 100026).
- *Cologuard* reagents are intended to be used only with the Exact Sciences System Software and instrumentation.
- To ensure the integrity of the sample, the laboratory must receive patient specimens within 72 hours of collection. Refer to *Cologuard Laboratory Procedure, Receipt of Cologuard Collection Kit* in this document.
- The barcoded identification numbers on the hemoglobin sample and the DNA sample from a Collection Kit must match for Hemoglobin and DNA assay results to be matched into an overall *Cologuard* result.
- Instrument and assay procedures reduce the risk of contamination during the laboratory procedure. However, good laboratory practice and careful adherence to the procedures specified in this document are important to reduce further risk of nucleic acid contamination from calibrators, positive controls, or specimens.
- Invalid results could occur from improper handling or storage, technical error, or sample mix up. Ensure that only trained personnel perform the laboratory procedure.
- *Cologuard* results are qualitative. The numeric value of the *Cologuard* Score is not indicative of extent of disease.
- A negative test result does not exclude the possibility that the patient may have a pre-cancerous or cancerous polyp. A false negative result with *Cologuard* could potentially delay colonoscopy and a potentially delayed diagnosis of disease.
- A positive *Cologuard* test suggests the presence of pre-cancerous polyps and/or cancer. A false positive result could result in an additional invasive screening procedure for the patient, such as colonoscopy, and thus expose patients to the attendant risks associated with such a procedure.
- Results from the *Cologuard* cross-contamination analysis indicated no observed cross-

contamination from automated equipment or repeated testing of manual steps. However, operator-induced cross-contamination can occur if procedures are not carefully followed.

- Cancers in organs connected to the digestive tract (i.e., pancreas and liver) may shed markers that could be detected by *Cologuard*. As such, it is expected that a certain level of reactivity will be observed in cases of these cancers. Refer to *Performance Characteristics, Sensitivity and Cross-Reactivity* in this document.

Performance Characteristics

Clinical Cutoff

The cut-offs and the algorithm for the *Cologuard* sDNA-based colorectal cancer screening test were established based on an evaluation of a panel of donor samples that were categorized by colonoscopy. Variable selection for the *Cologuard* model was performed as a stepwise selection with the main variables assessed one at a time based on their respective statistical significance. The total sample size of the dataset for algorithm development included 953 samples, including 794 normal samples, 73 advanced adenomas and 86 cancers. The derived *Cologuard* algorithm sensitivity and specificity compared to colonoscopy outcome demonstrated a sensitivity of approximately 98% for cancer and approximately 57% for advanced adenoma.

Analytical Sensitivity

Sensitivity: Limit of Blank (LoB), Limit of Detection (LoD), Limit of Quantification (LoQ) and Linearity.

LoB, LoD, and LoQ studies were performed for both the methylation and mutation component (i.e., molecular assay) and the hemoglobin assay component of *Cologuard* based on guidance from the CLSI Standard: EP17-A (*Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline*). For molecular assays, such as the *QuARTS* component of *Cologuard*, the signal from the blank wells is absent. Therefore, the LoD and LoQ were established through means independent of a Limit of Blank (LoB) measurement. For *NDRG4*, *BMP3*, *KRAS* 38A, *KRAS* 35T, and *ACTB*, a minimum of 60 replicates per marker near the LoD concentration were tested across 6 samples at the expected LoD concentration within a dilution series in order to use probit analysis to predict LoD. For LoQ, a minimum of 60 replicates per marker near the anticipated LoQ concentration was tested across 6 samples and the lowest concentration with total error less than that of the total error goal of 20% CV on log strands was the determined LoQ.

Linearity and Linear Range studies using concentrations above and below the anticipated linear range were tested in the molecular assay and hemoglobin assay components of *Cologuard*. Linearity studies were performed based on guidance from CLSI Standard: EP6-A (*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline).* All markers were individually assessed at 9 levels spanning 5 logs including concentrations 30% above and below the anticipated upper LoQ and LoD, respectively. The markers were tested at 8 replicates total per level per marker, 2 replicates per plate, 2 instruments with 1 operator per instrument. The 9 concentrations were (in log strands per reaction): 5.59, 5.48, 5.30, 4.30, 3.30, 2.30, 1.30, 1.00, and 0.85

In summary, analytical sensitivity characteristics for *Cologuard* were observed as follows (**Table 1**): The methylation markers NDRG4, BMP3, and BT-ACT have a LoD at 0.702-0.738 log strands. KRAS was assigned a LoD value to that of KRAS 35T, the KRAS mutant with the highest LoD. The KRAS LoD is 1.058 log strands with a CI range that encompasses the lower cutoff used in the *Cologuard* software. The established LoD meets criteria of the ability to detect one percent mutation or methylation when 3.000 log strands of ACTB are present and the LoD is less than or equal to 1.300 log strands. The molecular assay LoQ is 1.176 log strands per reaction. This exceeds the input requirement that the LoQ must be less than 2.000 log strands. The molecular assays demonstrate good linearity over at least 5 logs and that R^2 is = $\geq$ 0.996 for all targets.

Analytical sensitivity characteristics for *Cologuard* were observed as follows:

Table 1: Analytical Sensitivity Characteristics Summary

Performance Characteristic	Molecular Assay	Hemoglobin Assay
Limit of Blank	Not Applicable	0.4 ng/mL
Limit of Detection	Methylation Markers: *NDRG4, BMP3* and *ACTB* 0.702 to 0.738 log strands Mutation Markers: *KRAS* 1.058 log strands	1.3 ng/mL
Limit of Quantification	LoQ ≤ 1.176 log strands	4.8 ng/mL
Assay linearity	R^2 = $\geq$ 0.996 Linear range = 1.1760 to 5.591 log strands	Linear range = 4.8 ng/mL to 500 ng/mL No hook effect observed for concentrations up to 100 µg/mL

Specificity and Cross-Reactivity

Cologuard Molecular Assay Cross-Reactivity with Wild-Type KRAS

The potential for cross-reactivity with wild-type *KRAS* was evaluated by testing two levels of *KRAS* wild-type DNA in the *Cologuard QuARTS* methylation and mutation assays. *KRAS* wild-type DNA was assessed at levels of 20,000 copies of wild-type *KRAS*, which is greater than the average expected to be seen in normal human stool samples, and 200,000 copies of wild-type *KRAS*, 10 times higher.

Results from this study indicated that cross-reactivity for wild-type *KRAS* at 200,000 copies was nearly 0% for the methylation assay and 0.01% for the mutation assay.

Cologuard QuARTS Partial Methylation Testing

Many genes have elevated methylation in their promoter region in colorectal cancer, whereas the same genes have low levels of methylation in normal colon epithelial cells. The DNA oligonucleotides used in the *Cologuard* methylation assay are designed to be a perfect match to fully methylated DNA in *NDGR4* and *BMP3*.

The analytical specificity of the DNA methylation assay component of *Cologuard* was tested against partially methylated *BMP3* and *NDRG4* DNA targets using the *QuARTS* assay. The testing utilized synthetic DNA targets that contained all possible permutations of partial methylations in the *QuARTS* assay footprint region of *BMP3* and *NDRG4*.

The study results demonstrated that *Cologuard* is specific for highly methylated DNA, specifically highly methylated *NDRG4* and *BMP3*. At least five of eight potential methylation sites for *BMP3* and five of nine potential methylation sites for *NDRG4* have to be methylated for any reactivity in *Cologuard*. With respect to *NDRG4*, the percent cross-reactivity was 2.5%, indicating that the analytical specificity for total methylations in *NDRG4* is 97.5%. With respect to *BMP3*, the percent cross-reactivity was 1.8%, indicating that the analytical specificity for total methylations in *BMP3* is 98.2%, above the 95% specificity outlined in the pre-defined acceptance criteria.

***Cologuard* Hemoglobin Assay Cross-Reactivity and Specificity**

The ability of the Hemoglobin Assay to detect hemoglobin in specimens heterozygous for Hemoglobin S (HbS) and Hemoglobin C (HbC) was evaluated. Samples used for testing Hb variants consisted of a stool sample background spiked with normal, HbS heterozygous, or HbC heterozygous whole blood. The Hemoglobin Assay detected both HbS and HbC variants, when comparing equivalent volumes of blood from normal and heterozygous variant specimens.

Additionally, cross-reactivity of *Cologuard* Hemoglobin Assay with animal hemoglobin and myoglobin was evaluated. Samples used for testing animal blood cross-reactivity consisted of a stool sample background spiked with animal whole blood. Samples used for testing myoglobin cross-reactivity consisted of a stool sample background spiked with prepared meat extracts or purified myoglobin. Thirteen replicates of each sample type were tested with the *Cologuard* Hemoglobin Assay.

Included in the study were 21 samples: 6 human blood samples, 8 animal blood samples, 4 animal myoglobin samples prepared from meat extracts, 2 animal myoglobin samples from purified myoglobin, and 1 negative sample.

Mean Hb concentrations for all animal hemoglobin and myoglobin samples were less than the limit of detection (LoD) of the assay (1.3 ng/mL) after the mean concentration of the Hb Negative Stool Sample was subtracted, indicating that no cross-reactivity was detected.

***Cologuard* Cross-Reactivity with Non-Colorectal Cancers and Diseases**

The potential for cross-reactivity with non-colorectal cancers was evaluated by testing 151 specimens from subjects with cancers or diseases other than CRC that have a potential association with the GI tract, or inflammatory conditions that could affect the screening population for *Cologuard*. Samples were tested with both the Molecular and Hemoglobin Assay components of *Cologuard*. Overall *Cologuard* Scores were then generated to assess whether reactivity was found with any of these non-CRC samples.

Cancers in organs connected to the digestive tract (i.e., pancreas and liver) may shed markers that could be detected by *Cologuard*. As such, it is expected that a certain level of reactivity will be observed in cases of these cancers. The results of this testing are included in Table 2 below.

Table 2: Incident Rates and Contribution to *Cologuard* Positivity for Non-CRC Diseases and Cancers

Disease or Cancer*	Number of specimens tested	Incident rate per 10,000**	% Positivity of Cologuard	Number positive Cologuard calls in 10,000 subjects
Bladder Cancer	17	2.3	17.6%***	0.4
Breast Cancer	14	12.4	0.0%***	0.0
Esophagus Cancer	11	0.5	18.2%***	0.1
Gynecologic Cancer	11	2.0	36.4%	0.7
Hepatic Cancer	6	0.8	50%	0.4
IBD	18	1.0	38.9%	0.4
Lung Cancer	10	6.5	20.0%***	1.3
Lupus	17	0.2-0.8	11.8%***	0.1
Pancreas Cancer	12	1.2	41.6%	0.5
Prostate Cancer	12	15.5	8.3%***	1.3
Rheumatoid Arthritis	15	4.1	26.7%***	1.1
Stomach Cancer	8	0.8	25.0%***	0.2
Total per 10,000 subject		NA	NA	6.5

*Listed value for gynecologic cancer is the sum of ovarian and cervix uteri cancers.
**For cancers, figures were obtained from the National Cancer Institute (http://seer.cancer.gov/statfacts/index.html). For other diseases, figures were obtained from the Centers for Disease Control and Prevention (http://www.cdc.gov).
*** Not significantly greater than what would be expected in a "normal" population.

Based on the results of this study, considering the non-CRC diseases and cancers where the percent positivity was slightly higher than would be expected in a normal population, the expected positivity for the tested diseases would result in only a minimal (0.02%) decrease in specificity for Cologuard (or two positive calls per 10,000 screening patients tested).

Precision and Reproducibility

A laboratory-to-laboratory precision and reproducibility study was performed to assess variation of the *Cologuard* assay measurement system based on guidance from the CLSI Standard: EP15-A2 (*User Verification of Performance for Precision and Trueness; Approved Guideline*). As part of the study, a variance component analysis was performed by sample type for the *Cologuard* system to estimate the components of precision for each source of variation (operator, run, site, and replicate) as well as total variation for each individual marker and the overall *Cologuard* Score.

The study was performed at three sites (100, 200, 300), with a minimum of two operators at each site. A total of 22 *Cologuard* runs were performed at each site, 11 per operator. Each run involved 42 samples, including six replicates of each of the following: four stool pool samples (negative, high negative, low positive and high positive) and three control samples (negative, low positive and high positive).

For the molecular assay component of *Cologuard*, the stool sample types were prepared by combining characterized residual stool samples. The samples were characterized as positive or negative for CRC based on colonoscopy results. Subsequently, these residual clinical stool specimens were tested with the *Cologuard* assay and combined to establish the planned DNA content of samples for use in this study. Spiked synthetic DNA was used to create the contrived control samples.

For the hemoglobin assay component of *Cologuard*, the clinical stool pools were prepared by adding fresh whole blood to normal patient stool pools. Specifically, whole blood was spiked into stool samples and diluted to the appropriate concentration. Control samples (including negative, low, and high controls) were provided to each testing site in lyophilized form for reconstitution prior to testing.

Percent agreement between sites was evaluated by generating two-by-two (2 x 2) contingency tables for negative and positive results for all site pairs, calculating the average positive agreement (APA) and average negative agreement (ANA), and calculating the exact two-sided lower 95% confidence interval by the Clopper-Pearson method. The resulting lower confidence limit was then compared to the target agreement rate of 0.95. The lower confidence interval for percent agreement of all site pairs was ≥0.95. Inter-site agreement is shown in Table 3 and shows minimal variation.

Table 3: Inter-site Agreement

Site Comparison	Number Agreed	Total Compared	Agreement Rate	95% CI Lower Bound***
ANA* – Site 100 and Site 200	768	777	0.988	0.978
APA** – Site 100 and Site 200	1026	1035	0.991	0.983
Site Agreement – Site 100 and Site 200	897	906	0.990	0.982
ANA – Site 100 and Site 300	744	746	0.997	0.990
APA – Site 100 and Site 300	1012	1014	0.998	0.993
Site Agreement – Site 100 and Site 300	878	880	0.998	0.992
ANA – Site 200 and Site 300	756	764	0.990	0.979
APA – Site 200 and Site 300	1004	1012	0.992	0.984
Site Agreement – Site 200 and Site 300	880	888	0.991	0.982

*ANA = Average negative agreement
**APA = Average positive agreement
***Clopper-Pearson Confidence Interval

Descriptive statistics were separately calculated for all marker/sample combinations. %CV was calculated only for samples with an expected positive result. Inter-site descriptive statistics are provided below (Table 4).

Table 4: Inter-Site Descriptive Statistics for the *Cologuard* Score

Sample	N	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Std Dev	Total %CV
Negative Stool Pool	387	9.98	9.65	10.31	3.31	NA
High Negative Stool Pool	394	62.92	60.24	65.61	27.14	NA
Low Positive Stool Pool	393	391.11	383.66	398.36	74.13	18.96
High Positive Stool Pool	394	978.34	977.44	979.24	9.13	0.93
Negative Control	392	6.35	6.26	6.44	0.90	NA
Low Positive Control	393	626.24	621.39	631.09	48.91	7.81
High Positive Control	393	963.38	962.30	964.46	10.89	1.13

Overall, the assay was highly reproducible with inter-site agreement values of the lower confidence interval of >95% (Table 4) and all of the positive *Cologuard* Scores had inter-site CVs of less than 20% (Table 5).

An additional multi-operator prospective study was conducted to further evaluate the intermediate precision and repeatability of the processes developed for use with the *Cologuard* assay with high negative and low positive stool samples containing levels of DNA or hemoglobin that together, give a *Cologuard* Score near the cut-off of the *Cologuard* assay. The results from the supplemental precision and reproducibility study demonstrated acceptable %CV for samples near the assay cut-off.

The study was performed at one site with two operator teams. A total of 22 *Cologuard* runs were performed during the study, in which each operator team performed 11 complete runs, with each run requiring 2 shifts to complete. Each run involved 12 samples, including six replicates of each of the high negative and low positive stool samples. A single lot of *Cologuard* reagents and controls was used throughout the study.

Percent agreement between operators was evaluated by generating two-by-two (2 x 2) contingency tables for negative and positive results, calculating the weighted average negative agreement (ANA) and average positive agreement (APA), and calculating the exact two-sided lower 95% confidence interval. The lower confidence interval for total agreement of all pairs was >0.95% agreement of all site pairs. Descriptive statistics were calculated and are shown in Table 5 below.

Table 5: Inter-Operator Descriptive Statistics for the *Cologuard* Score

Sample	N	Median	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Std Dev	%CV
High Negative Stool Pool	132	141.9	142.0	139.1	145.0	17.4	12.2
Low Positive Stool Pool	132	238.5	236.5	232.7	240.2	21.8	9.2

Overall the additional testing continued to demonstrate that the assay was highly reproducible with inter-operator agreement values of the lower confidence interval of >95% and all of the positive *Cologuard* Scores had inter-operator CVs of less than 20%.

Lot-to-Lot Reproducibility

Lot-to-Lot reproducibility was evaluated for *Cologuard* based on guidance from the CLSI Standards: EP5-A2 (Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline); EP15-A2 (User Verification of Performance for Precision and Trueness; Approved Guideline); EP12-A2 (User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline); and I/LA28-A2 (Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays; Approved Guideline).

Lot-to-Lot reproducibility was assessed by testing a sample panel comprised of seven samples containing various levels of DNA and hemoglobin, using three lots of *Cologuard* reagents and controls.

For the molecular assay component of *Cologuard*, the stool sample types were prepared by combining characterized residual stool samples available to Exact Sciences. The samples were characterized as positive or negative for CRC based on colonoscopy results. Subsequently, these residual clinical stool specimens were tested with the *Cologuard* assay and combined to establish the planned DNA content of samples for use in this study. Spiked synthetic DNA was used to create the contrived control samples.

For each sample in the panel, there were 24 sample results per lot and 72 sample results for the entire study. Across the seven samples in the panel, there were 168 results per lot, and 504 results for the entire study.

The mean, SD, %CV, N, minimum value and maximum value were calculated for each marker or each lot and test sample. Additionally, *Cologuard* Scores were determined. Percent positive results for the *Cologuard* Score were analyzed across lots and for lot to lot. Variance component analyses were also conducted.

Descriptive statistics were calculated for all marker/sample combinations, including median, mean, mean upper and lower 95% confidence intervals, standard deviation, and coefficient of variation values. %CV was calculated only for controls with expected result of positive. Descriptive statistics were calculated both within and across lots. Descriptive statistics for this study are shown below (Table 5). The *Cologuard* Score %CV values for positive samples were within the pre-specified acceptance criteria, ranging between 0% and 16.8%.

Table 6: Descriptive Statistics for Lot-to-Lot Cologuard Score

Sample Name	N	Median	Mean	Lower 95% CL for Mean	Upper 95% CL for Mean	Std Dev	CV
Negative Stool Pool	72	9.47	11.39	10.19	12.58	5.07	NA
High Negative Stool Pool	72	64.46	57.74	51.12	64.36	28.18	NA
Low Positive Stool Pool	71	380.75	373.93	359.03	388.84	62.98	16.84
High Positive Stool Pool	71	973.92	972.88	970.36	975.40	10.64	1.09
Negative Control	70	6.33	6.40	6.21	6.59	0.79	NA
Low Positive Control	71	584.09	579.52	570.09	588.95	39.85	6.88
High Positive Control	71	1000	1000	1000	1000	0	0

Percent agreement between lots was evaluated by generating 2 x 2 tables for negative and positive results for all lot pairs, calculating the average positive agreement (APA) and average negative agreement (ANA). Testing of samples with various levels of hemoglobin and DNA markers demonstrated a percent agreement for positive and negative samples across multiple lots between 98.6% and 100%, with a lower confidence limit above 95% (Table 6).

Table 7: Lot-to-Lot Percent Agreement

Lot Comparison	Number Agreed	Total Compared	Agreement Rate *	95% CI Lower Bound***
ANA* - Lot1 and Lot2	142	142	1.0000	0.9744
APA** - Lot1 and Lot2	188	188	1.0000	0.9806
Lot Agree - Lot1 and Lot2	165	165	1.0000	0.9779
ANA - Lot1 and Lot3	140	142	0.9859	0.9501
APA - Lot1 and Lot3	180	182	0.9890	0.9609
Lot Agree - Lot1 and Lot3	160	162	0.9877	0.9561
ANA - Lot2 and Lot3	142	144	0.9861	0.9507
APA - Lot2 and Lot3	184	186	0.9893	0.9617
Lot Agree - Lot2 and Lot3	163	165	0.9879	0.9569

NOTE: Proportion values are point estimates used to determine the Clopper-Pearson 2-sided Confidence Interval. Only Clopper-Pearson Lower Limit values are shown in the above table.
*ANA = Average negative agreement
**APA = Average positive agreement
***Clopper-Pearson Confidence Interval

The study demonstrated that *Cologuard* results are reproducible across multiple reagent lots.

Robustness

The *Cologuard* performance was assessed in response to defined variable factors (see below) at specific steps in the test procedure, using both the molecular assay and hemoglobin assay components of *Cologuard*. The processing steps analyzed in this study are the steps at which operator variability or error are most likely to occur. Three total instrument and operator sets were used for the study.

Cologuard Molecular Assay Robustness

Results when these various factors were introduced into the processing steps were compared to the expected results for a positive stool sample, a control sample with high levels of mutation and methylation markers, and a control sample with moderate levels of mutation and methylation markers. Fourteen replicates of each sample type were used. Analysis of these samples assumed a hemoglobin value of zero, when calculating overall *Cologuard* score. Factors tested included the following:

- Factors related to DNA capture, including wait times between processing steps, amount of reagents added, and duration of storage at the appropriate temperatures;
- Factors related to the amount of time various instruments are paused during the automated DNA preparation and *QuARTS* assay steps of the *Cologuard* process; and
- Factors related to the amount of time between plate assembly and processing during the *QuARTS* assay step.

The results for the molecular assay component of *Cologuard* showed that time between plate assembly and processing during the *QuARTS*™ assay step and the number of days the captured DNA was stored at the appropriate temperatures could have a detectable effect on

assay response. Testing demonstrated that the prepared *QuARTS*™ plate should be processed within 30 minutes and captured DNA could be tested for up to four days.

Cologuard Hemoglobin Assay Robustness

Results when these factors were introduced into the processing steps were compared to the expected results for a stool sample with a known level of endogenous hemoglobin and a high and low control sample with high and low levels of hemoglobin. The study tested 16 replicates of each sample type. Analysis of these results involved comparing the resulting hemoglobin concentration with the expected hemoglobin concentration. Factors tested include the following:

- Time between steps during plate preparation;
- Incubation times for antibodies and substrates; and
- Time between steps during plate reading phase.

Results for the hemoglobin assay component of *Cologuard* showed that substrate incubation time had a detectable effect on assay performance. Testing demonstrated that a substrate incubation time of 15 ± 1.5 minutes would result in acceptable assay performance.

Interfering Substances

***Cologuard* Molecular Assay Interference Testing**

Interference with the molecular assay component of *Cologuard* was evaluated using 55 common substances that potentially could be present in stool materials including potential interfering substances in the following categories:

- Common lotions, creams, and feminine over-the-counter products;
- Stool softeners, anti-diarrhea, and laxative products;
- Anti-acids and upset stomach relief products;
- Animal genomic DNA of commonly edible animals (both high and low levels);
- Urine and alcohol;
- A mixture of common vegetables and fruits; and
- Fecal Fats (fatty acids and cholesterol).

No interference with the molecular assay component of *Cologuard* was observed for any of the tested substances.

***Cologuard* Hemoglobin Assay Interference Testing**

Interference with the Hemoglobin Assay component of *Cologuard* was evaluated using 49 common substances that potentially could be present in stool materials including potential interfering substances in the following categories:

- Common lotions, creams, and feminine over-the-counter products;
- Urine;
- Stool softeners, anti-diarrhea, and laxative products;
- Anti-acids and upset stomach relief products;
- Antibiotics, anti-inflammatories, anti-fungal drugs, pain relievers, and decongestants;
- A mixture of common vegetables and fruits;

- Fats and lipids;
- Alcohol;
- Iron sulfate (as found in oral supplements);
- Vitamin C; and
- DNA Stabilization Buffer (preservative solution provided in the *Cologuard* Collection Kit for the whole stool sample used in the molecular assay).

None of the substances tested interfered with the *Cologuard* hemoglobin assay.

Carry-over and Cross-contamination Cologuard Testing

Carry-over Evaluation

Sequential runs of high positive and negative samples were used to evaluate carry-over contamination for each assay component of *Cologuard*. Testing of the molecular assay and hemoglobin assay components was conducted in two separate studies.

For the molecular assay (methylation/mutation assay), the testing involved two consecutive runs of high positive DNA samples, composed of 10x high level run controls diluted in Tris, EDTA and non-human DNA, followed by a run of negative samples composed of Tris, EDTA and non-human DNA. A total of 43 high positive samples and 3 run controls were used in each high positive run. A total of 43 negative samples and 3 run controls were used for the negative run.

For the hemoglobin assay, the testing involved two consecutive runs of high positive hemoglobin samples, composed of 100,000 ng/mL hemoglobin, followed by a run of negative samples composed solely of the protein preservative solution from the hemoglobin sample collection tube. The high positive samples consisted of a hemoglobin level that is much higher than the quantitative range of the assay, which identifies all samples >500 ng/mL as greater than the maximum range of the assay. For the high positive runs, a total of 86 high positive hemoglobin samples were used. For the negative run, 86 negative samples were used. In each run, the signal obtained on the controls was utilized to ensure the validity of the run.

Results from the molecular assay and hemoglobin assay carry-over analyses demonstrated that there was no carry-over in the *Cologuard* assay.

Cross-contamination Evaluation

Cross-contamination testing of *Cologuard* was based on a checkerboard study design, alternating high positive and negative samples, to evaluate the potential for contamination from the positive to the negative samples within a run. Testing of the molecular assay and hemoglobin assay components was conducted in two separate studies.

For the molecular assay, 22 high positive samples, 21 negative samples, and three run control samples were used. The high positive samples for this study were composed of 10x high level run controls diluted in Tris, EDTA and non-human DNA, and the negative samples were composed of Tris, EDTA and non-human DNA. One run was performed and samples were processed using the *Cologuard* molecular process from the semi-automated front end sample processing through the automated processing.

For the hemoglobin assay, a total of 43 high hemoglobin and 43 negative hemoglobin samples were used. As in the carry-over study, the high positive samples contained 100,000 ng/mL

hemoglobin, while the negative samples consisted solely of the protein preservative solution from the hemoglobin sample collection tube. Three runs were performed and samples were processed using the *Cologuard* hemoglobin process.

Results from the cross-contamination analysis for the molecular assay demonstrated that the molecular assay component of *Cologuard* and the associated instruments needed to run the assay performed as intended and met the study acceptance criteria. Specifically, one well experienced some cross-contamination (52 strands of ACTB), however, this was within the pre-specified acceptance criteria, which dictated that no more than three wells could exhibit 10-100 strands of ACTB and no single well could exhibit more than 100 strands.

The high hemoglobin samples utilized in this study contained hemoglobin levels that are approximately 50 times higher than the median positive hemoglobin values observed in colorectal cancer subjects (Levi et. al, 2007). The high hemoglobin concentrations tested in this study are much higher than would be expected in use of *Cologuard*.

Testing of the Hemoglobin Assay cross-contamination showed a low level of contamination (~0.01%). Signal was observed in 4 out of 43 negative samples with an average detectable hemoglobin level of 11 ng/mL (0.011%). This calculates to a 0.011% contamination level in those four samples. As the hemoglobin assay involves several manual steps (e.g., manual washing and reagent addition), repeat testing was conducted, in which no cross contamination was observed.

Under normal use conditions, low level contamination observed in this study would be negligible. However, this study provides evidence that cross contamination is possible due to the manual steps in the assay processing.

Stability Studies

In-Use Stability: Molecular Assay Stability Under Standard Operating Conditions

The stability of reagents used in the molecular assay component of *Cologuard* was evaluated following guidance from CLSI standard: EP25-A (*Evaluation of Stability of In Vitro Diagnostic Reagents; Approved Guideline*). The purpose of this testing was to determine reagent stability after opening the containers and using them under potential user operating conditions. All reagents required for the molecular assay were tested.

Samples were processed with the molecular assay component of *Cologuard*, using these reagents, to determine the in-use stability of the reagents and the effect of the various factors on *Cologuard* results. The samples used in the in-use stability study for the various *Cologuard* reagent groups included DNA calibrators; High Positive and Low Positive control samples consisting of synthetic targets in stool collection buffer; a Negative DNA control sample; DNA positive and negative run controls; and a positive stool sample.

The study demonstrated that *Cologuard* reagents are stable when opened or stored for variable times before use under standard operating conditions. Specifically:

- Multiple-use reagents stored at room temperature are stable for up to six weeks from the open date.

- Capture Beads that have been pre-washed and stored at 2-8°C are stable for up to 13 days.
- Pre-washed Capture Beads are stable for up to six hours at room temperature prior to use.

Single-use reagents that are used on the automated system are stable on the Hamilton Microlab® STARlet deck for up to 4 hours prior to the start of the run.

Freeze-Thaw Stability

A freeze-thaw stability study was conducted to evaluate the stability of the *QuARTS*™ assay reagents when subjected to repeated freeze/thaw events. The *QuARTS*™ assay reagents tested included only those assay components normally stored frozen (-25 to -15°C):

1) Oligo Mix A, Methylation;

2) Oligo Mix B, Mutation;

3) Enzyme Mix;

4) DNA Calibrator 1 High Methylation;

6) DNA Calibrator 2 Low Methylation;

7) DNA Calibrator 3 High Mutation; and

8) DNA Calibrator 4, Low Mutation.

Materials from one lot of each assay component were subjected to 0, 2, 4, and 6 freeze-thaw cycles. Each component was then tested in the *Cologuard* molecular assay component using the *Cologuard* DNA Controls (i.e., DNA Control 1, High Positive and DNA Control 2, Low Positive), which did not undergo freeze-thaw cycling. The study tested 16 replicates for each component and each freeze-thaw cycle. Calibrators used during testing to assess assay validity and to generate curves for sample concentration assessment were not subjected to freeze-thaw cycling. Log strands for each marker were compared to those for samples where the reagents did not undergo freeze thaw cycling.

All log strand results for all samples were statistically equivalent to those that did not undergo freeze thaw cycling, thereby demonstrating that the *Cologuard QuARTS*™ assay reagents are stable for six freeze thaw events.

Real-Time Stability

Real-time stability testing of *Cologuard* was conducted by evaluating the functional performance of three reagent lots over a period of 41 weeks. Each lot was comprised of unique batches of reagents, which were tested at various time points over 41 weeks.

Samples that were used to evaluate hemoglobin assay reagent stability consisted of negative stool matrix spiked with whole blood to create samples with a low and high hemoglobin concentration. Samples for evaluation of molecular assay reagent stability consisted of negative stool matrix spiked with oligonucleotides that contain the marker sequences. Oligonucleotides for *NDRG4, BMP3, BTACT, KRAS1, KRAS2,* and *ACT* were spiked into the negative stool

samples to create samples with a low and high level of sDNA samples. At each time point, seven replicates of samples and controls were tested.

The results of the real time stability studies demonstrated that overall the components of the *Cologuard* assay gave similar results through the 41 week study. These data supported the 6 month shelf life currently assigned to the *Cologuard* assay reagents.

Clinical Sensitivity and Specificity

The pivotal study ("Multi-Target Colorectal Cancer Screening Test for the Detection of Colorectal Advanced Adenomatous Polyps and Cancer: DeeP-C Study") was conducted to generate data to support the safety and effectiveness of *Cologuard* as a screening test for the detection of markers associated with the presence of colorectal cancer (CRC) and advanced adenoma (AA). To evaluate the performance of *Cologuard*, the *Cologuard* test result (negative or positive) was compared with the histopathological result from optical colonoscopic examination and histopathological diagnosis of all significant lesions discovered during the colonoscopy and either biopsied or removed. Based on this comparison, *Cologuard* sensitivity (true positive fraction) was 92.3% (60/65) for subjects with a histopathological diagnosis of CRC and 42.4% (322/760) for subjects with a diagnosis of AA. For subjects without a diagnosis of CRC or AA, *Cologuard* specificity (true negative fraction) was 86.6% (7967/9198). Furthermore, among subjects having a valid *Cologuard* test result and also a valid test result from a commercially available FIT (OC FIT-CHEK, Polymedco, Inc.) ("FIT"), both sensitivity for CRC and sensitivity for AA were higher for *Cologuard* (92.3%, 42.4%) than for FIT (73.8%, 23.8%), and both differences (18.5%, 18.6%) were significantly different from zero (p=0.002, 0.001). However, for subjects without CRC or AA, specificity was lower for *Cologuard* (86.6%) than for FIT (94.9%), and the difference (-8.3%) was significantly different from zero ($p < 0.0001$).

An overview of the study design and results is provided below.

Study Design

The *Cologuard* pivotal study was a prospective, cross-sectional, multi-center study (DeeP-C study) that began enrollment of study participants on June 30, 2011. A total of 12,776 patients were enrolled from 90 sites in the U.S. and Canada, including both colonoscopy centers and primary care sites, with study participation concluding on February 4, 2013. Subjects were provided with a collection kit, which they used to collect stool samples for *Cologuard* and FIT testing. Subjects subsequently underwent colonoscopy within 90 days of study enrollment.

The stool samples for analysis with *Cologuard* were sent to a central biorepository for batch testing at one of three laboratories while the stool samples for the FIT were sent to a single laboratory for testing. Samples tested with *Cologuard* were assayed by laboratory technicians blinded to the results of colonoscopy and the FIT results. Results from *Cologuard* and the FIT test were compared to the results of an optical colonoscopic examination, and histopathological diagnosis of all significant lesions discovered during the colonoscopy and either biopsied or removed.

Colonoscopy findings were recorded per site specific standard of practice. Subjects with no findings were categorized as negative by colonoscopy. Histopathological results from biopsied tissue or excised lesions were categorized based on the most clinically significant lesion present (i.e., the index lesion) by a central pathologist according to the pre-specified standards outlined in **Table 8**.

Table 8: Histopathological category definitions

Category	Findings
1	CRC, all stages (I-IV)
2	Advance adenoma, including the following subcategories: 2.1 – Adenoma with carcinoma *in situ*/high grade dysplasia, any size 2.2 – Adenoma, villous growth pattern (≥25%), any size 2.3 – Adenoma ≥ 1.0 cm in size, or 2.4 – Serrated lesion, ≥ 1.0 cm in size
3	1 or 2 adenoma (s), >5 mm in size, or < 10 mm size, non-advanced
4	≥ 3 adenomas, <10mm, non-advanced
5	1 or 2 adenoma(s), ≤5 mm in size, non-advanced
6	Negative – No neoplastic findings 6.1 – negative upon histopathological review 6.2 – no findings on colonoscopy, no histopathological review

Inclusion and Exclusion Criteria

Subjects eligible for enrollment in the study were of both genders between the ages of 50 and 84 years (inclusive), who were at average risk for development of colorectal cancer and asymptomatic for gastrointestinal symptoms warranting diagnostic colonoscopy. In addition, subject enrollment was age-weighted toward a slightly older population to increase the point prevalence of colorectal cancer in this study. An effort was made to enroll the majority of subjects of age 65-84; 64% of subjects in the actual study population were of age 65-84.

Clinical Performance Measures

The performance of *Cologuard* was evaluated based on comparison of the test result with the histopathological category (Table 9 above). Two co-primary performance measures were pre-specified: *Cologuard* sensitivity for subjects diagnosed with CRC (histopathological category 1), and *Cologuard* specificity for subjects without a diagnosis of CRC or AA (categories 3-6). For subjects with CRC, *Cologuard* sensitivity is the fraction of CRC subjects called positive by the *Cologuard* test (true positive fraction). Defining advanced neoplasia (AN) as CRC or AA, *Cologuard* specificity for AN is the fraction of non-AN subjects called negative by the *Cologuard* test (true negative fraction). For the study to be successful, the co-primary analysis required that the lower bound of the 95% one-sided confidence interval was greater than 65% for *Cologuard* sensitivity for CRC and greater than 85% for *Cologuard* specificity for AN. It should be noted that sensitivity for CRC and specificity for AN are not complimentary in that advanced adenoma (AA, histopathological category 2) is left out of the definition of both measures.

Two secondary performance evaluations were pre-specified: *Cologuard* was evaluated for non-inferiority to FIT in sensitivity for CRC and for superiority to FIT in sensitivity for AA (fraction of AA subjects testing positive). Per the pre-specified protocol, *Cologuard* would be declared non-inferior to FIT in sensitivity for CRC if the one-sided 95% confidence interval lower bound on the *Cologuard* – FIT difference exceeded -5%. If *Cologuard* were to be declared non-inferior to FIT in CRC sensitivity, then evaluation for superiority to FIT in CRC sensitivity was permitted and declared if the difference was positive and its one-sided p-value (based on exact McNemar test) was less than 0.025. Likewise, per protocol, *Cologuard* would be declared superior to FIT in AA sensitivity if the *Cologuard* – FIT difference was positive and the one-sided McNemar p-value

was less than 0.025.

Accountability of PMA Cohort

The study enrolled a total of 12,766 subjects at 90 sites, including both primary care point-of-referral (POR) sites and colonoscopy centers. A total of 2,753 subjects were excluded from the primary analysis population due to unusable data (e.g., no colonoscopy). A total of 10,023 subjects were included in the primary analysis population. This population included 65 subjects with CRC. Analysis was conducted to rule out bias associated with the subjects excluded from the analysis population.

Study Population Demographics and Baseline Parameters

The baseline demographic characteristics for the Primary Effectiveness Population are presented in Table 9 below. As shown in the table, the average age of subjects was 64.2 years old, and there was a slightly higher percentage of female subjects (5,378/10,023, 53.7%) as compared with male subjects (4,645/10,023, 46.3%). The majority of subjects were White (8,422/10,017, 84.1%), although 10.7% of the population were Black or African American subjects (1,071/10,017). Nearly 10% of subjects were Hispanic or Latino (991/10,019, 9.9%). Average BMI was 28.83 and the majority of subjects never smoked (5,531 /10,019, 55.2%). It should be noted that two 49-year-old subjects and one 44-year-old subject were included in the study, which is inconsistent with the intended use population. Each of these subjects was a true negative and their inclusion did not notably impact data analyses.

Subjects that were enrolled at POR sites were similar to those enrolled at non-POR sites and to the population as a whole.

Table 9: Baseline Demographics – Primary Effectiveness Subjects

Parameter Statistic	All Enrolled (N=10023)	Specificity Subset (Cat. 2-6) (N=9958)	Specificity Subset (Cat. 3-6) (N=9198)	CRC Subset (Cat. 1) (N=65)	AA Subset (Cat. 2) (N=760)	*Cologuard*FIT Secondary Effectiveness (N=65)
Age (years) at Screening						
n	10023	9958	9198	65	760	65
Mean (SD)	64.2 (8.42)	64.1 (8.41)	64.0 (8.44)	70.2 (7.92)	65.4 (7.93)	70.2 (7.92)
Median	66	66	66	70	66	70
Min, Max	44, 84	44, 84	44, 84	50, 84	50, 84	50, 84
Gender, n (%)						
Male	4645 (46.3)	4611 (46.3)	4161 (45.2)	34 (52.3)	450 (59.2)	34 (52.3)
Female	5378 (53.7)	5347 (53.7)	5037 (54.8)	31 (47.7)	310 (40.8)	31 (47.7)
Race, n (%)						
White	8422 (84.1)	8367 (84.1)	7726 (84.0)	55 (84.6)	641 (84.5)	55 (84.6)
Black or African American	1071 (10.7)	1063 (10.7)	978 (10.6)	8 (12.3)	85 (11.2)	8 (12.3)
Asian	259 (2.6)	258 (2.6)	245 (2.7)	1 (1.5)	13 (1.7)	1 (1.5)
American Indian or Alaska Native	36 (0.4)	36 (0.4)	32 (0.3)	0 (0.0)	4 (0.5)	0 (0.0)
Native Hawaiian or Other Pacific Islander	23 (0.2)	23 (0.2)	23 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)
Other	206 (2.1)	205 (2.1)	189 (2.1)	1 (1.5)	16 (2.1)	1 (1.5)
Missing	6	6	5	0	1	0
Ethnicity, n (%)						
Hispanic or Latino	991 (9.9)	982 (9.9)	923 (10.0)	9 (13.8)	59 (7.8)	9 (13.8)
Not Hispanic or Latino	9028 (90.1)	8972 (90.1)	8272 (90.0)	56 (86.2)	700 (92.2)	56 (86.2)
Missing	4	4	3	0	1	0
BMI (kg/m2) at Baseline						
n	10015	9950	9190	65	760	65
Mean (SD)	28.83 (5.836)	28.84 (5.841)	28.77 (5.817)	27.55 (4.861)	29.67 (6.068)	27.55 (4.861)
Median	28.0	28.0	27.9	26.8	29.0	26.8
Min, Max	13.3, 68.2	13.3, 68.2	13.3, 68.2	19.3, 42.4	16.3, 59.9	19.3, 42.4
Smoking History, n (%)						
Never Smoked	5531 (55.2)	5498 (55.2)	5157 (56.1)	33 (50.8)	341 (44.9)	33 (50.8)
Former Smoker	3589 (35.8)	3564 (35.8)	3279 (35.6)	25 (38.5)	285 (37.5)	25 (38.5)
Current Smoker	903 (9.0)	896 (9.0)	762 (8.3)	7 (10.8)	134 (17.6)	7 (10.8)
If Former or Current Smoker, Daily Use, n (%)						
<1/2 Pack Per Day	2162 (48.3)	2154 (48.4)	1970 (48.9)	8 (25.0)	184 (44.0)	8 (25.0)
1 Pack Per Day	1585 (35.4)	1569 (35.3)	1418 (35.2)	16 (50.0)	151 (36.1)	16 (50.0)

Parameter Statistic	All Enrolled (N=10023)	Specificity Subset (Cat. 2-6) (N=9958)	Specificity Subset (Cat. 3-6) (N=9198)	CRC Subset (Cat. 1) (N=65)	AA Subset (Cat. 2) (N=760)	*Cologuard*FIT Secondary Effectiveness (N=65)
>1 Pack Per Day	732 (16.3)	724 (16.3)	641 (15.9)	8 (25.0)	83 (19.9)	8 (25.0)
Missing	13	13	12	0	1	0
If Former or Current Smoker, # Years Smoking						
n	4480	4448	4029	32	419	32
Mean (SD)	21.82 (14.733)	21.77 (14.732)	21.13 (14.450)	28.47 (13.488)	27.93 (15.959)	28.47 (13.488)
Median	20.0	20.0	20.0	29.0	30.0	29.0
Min, Max	0.0, 70.0	0.0, 70.0	0.0, 70.0	1.0, 60.0	1.0, 65.0	1.0, 60.0

Safety and Effectiveness Results

Primary Effectiveness Evaluations (Sensitivity for CRC/Specificity for AN)

The primary effectiveness population consisted of 10,023 subjects with a valid histopathological diagnosis and a valid Cologuard result. The basic data table for primary effectiveness evaluation is provided (Table 11).

Table 10: Distribution of *Cologuard* Result by Histological Category (%), n = 10,023

Cologuard Result	CRC (Category 1)	AA (Category 2)	Categories 3-6
Negative	5 (7.7)	438 (57.6)	7967 (86.6)
Positive	60 (92.3)	322 (42.4)	1231 (13.4)

The primary objectives of the DeeP-C study – demonstration of greater than 65% *Cologuard* sensitivity for CRC and greater than 85% *Cologuard* specificity for AN – were successfully met. Specifically, *Cologuard* sensitivity for CRC was 92.3% (60/65) with a one-sided 95% confidence interval lower bound of 84.5% (Table 11). *Cologuard* specificity for AN was 86.6%, with a one-sided 95% confidence interval lower bound of 86.0% (Table 12). Further, the two-sided 95% confidence interval was 83.0-97.5% for *Cologuard* CRC sensitivity and 85.9-87.3% for *Cologuard* AN specificity.

Table 11: Sensitivity for CRC – Primary Effectiveness Subjects with Valid *Cologuard* Positive Result (N=65)

Case Category	n/N (%)
1: CRC Stages 1-4	60/65 (92.3%)
Sensitivity Based on Category 1: Primary (one-sided 95% CI lower bound)	92.3% (>84.5%)
Sensitivity Based on Category 1: Supportive (one-sided 97.5% CI lower bound)	92.3% (>83.0%)

[1] Percentages based on valid test results within a category.
[2] Lower bounds calculated using an exact one-sided binomial test.

Table 12: Specificity for AN – Primary Effectiveness Subjects with Valid *Cologuard* Negative Result (N=9198)

Case Category	n/N (%)
3: 1-2 Adenomas 5 – <10 mm	607/749 (81.0%)
4: ≥3 Adenomas <10 mm, Non-advanced	302/419 (72.1%)
5: 1-2 Adenomas <5 mm, Non-advanced	1496/1735 (86.2%)
6.1: Negative upon histopathological review	1543/1821 (84.7%)
6.2: No findings on colonoscopy, no histopathological review	4019/4474 (89.8%)
Specificity Based on Categories 3-6: Primary (one-sided 95% lower bound)	86.6% (>86.0%)
Specificity Based on Categories 3-6: Supportive (one-sided 97. 5% lower bound)	86.6% (>85.9%)

[1] Percentages based on valid test results within a category.
[2] Lower bounds calculates using an exact one-sided binomial test.
[3] As noted above, one 44-year-old and two 49-year-old true negative subjects were included in the analysis population, although they would not be included in the intended user population.

Secondary Effectiveness Evaluations

The secondary effectiveness population consisted of 9,989 subjects with a valid histopathological diagnosis, a valid *Cologuard* result, and a valid FIT result. The basic data table for secondary effectiveness evaluation is provided (Table 13).

Table 13: Distribution of *Cologuard* and FIT Results by Histological Category, n = 9,989

CRC (Category 1)

	FIT	
Cologuard	**Negative**	**Positive**
Negative	4	1
Positive	13	47

AA (Category 2)

	FIT	
Cologuard	Negative	Positive
Negative	407	29
Positive	170	151

Categories 3-6

	FIT	
Cologuard	Negative	Positive
Negative	7787	149
Positive	908	323

Cologuard was compared with FIT for non-inferiority in sensitivity for CRC with respect to margin 5% and for superiority in sensitivity for advanced adenoma (AA). Secondary performance goals comparing *Cologuard* with FIT were evaluated in subjects having valid results from both tests.

Sensitivity for CRC was greater for *Cologuard* (92.3%, 60/65) than for FIT (73.8%, 48/65) (**Table 14 and Figure 1**), for a difference of 18.5%. *Cologuard* identified 13 CRCs that were missed by FIT. FIT identified one CRC that was missed by *Cologuard*. The one-sided 95% confidence interval lower bound on the *Cologuard* – FIT difference was 8.0%, which exceeds -5%, indicating that *Cologuard* is non-inferior to FIT in sensitivity for CRC with respect to the pre-defined non-inferiority margin 5%. Because *Cologuard* was declared non-inferior to FIT in sensitivity for CRC, it is statistically justifiable and was permissible per protocol to evaluate it for superiority to FIT as well. Sensitivity for CRC was significantly greater for *Cologuard* than for FIT (two-sided McNemar exact p value 0.0018), indicating that *Cologuard* is superior to FIT in sensitivity for CRC. Finally, for the *Cologuard* – FIT difference of 18.5% in CRC sensitivity, the two-sided 95% exact confidence interval was 7.2-30.4% (**Table 15**).

Table 14: Overall Sensitivity: CRC Subset (Category 1) - Secondary Effectiveness Subjects with Valid Results from Both *Cologuard* and FIT Tests (N=65)

	Cologuard	**FIT**
1: CRC Stages 1-4 **(n/N (%))**	60/65 (92.3%)	48/65 (73.8%)
Sensitivity Based on Category 1: Primary (one-sided 95% lower bound)	92.3% (>84.5%)	73.8% (>63.4%)
Sensitivity Based on Categories 1: Supportive (one-sided 97. 5% lower bound)	92.3% (>83.0%)	73.8% (>61.5%)

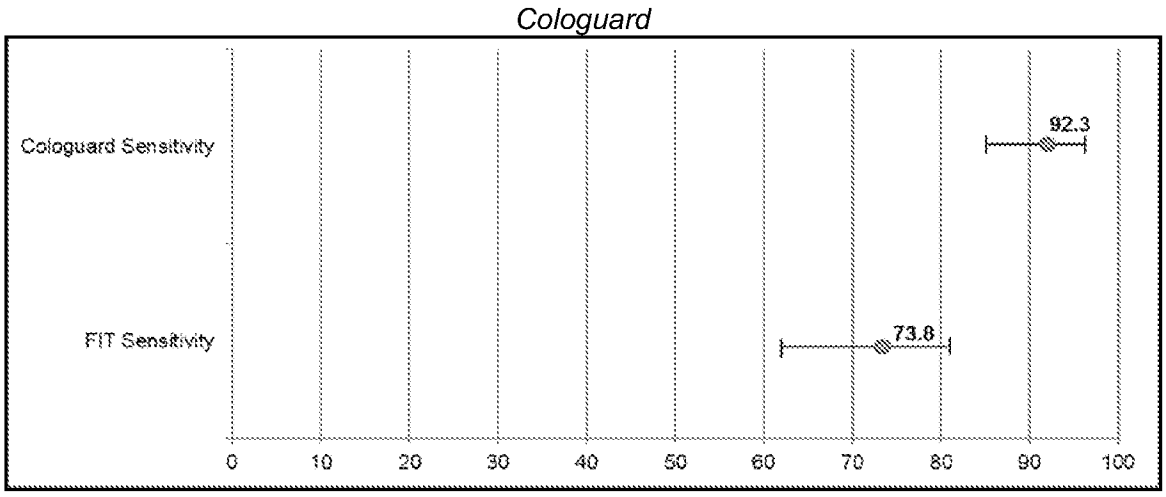
[1] Percentages based on valid test results within a category.
[2] Lower bounds calculated using an exact one-sided binomial test.

Table 15: Sensitivity Non-Inferiority and Superiority Test – CRC Subset (Category 1)

	FIT Outcome			**McNemar test p-value**
***Cologuard* Outcome**	**Negative**	**Positive**	**Totals**	
Negative, n (%)	4 (80.0)	1 (20.0)	5	0.0018
Positive, n (%)	13 (21.7)	47 (78.3)	60	
Totals	17	48	65	

[1] p-value is from a McNemar paired comparison test of the discordant pairs.

Figure 1: CRC Sensitivity



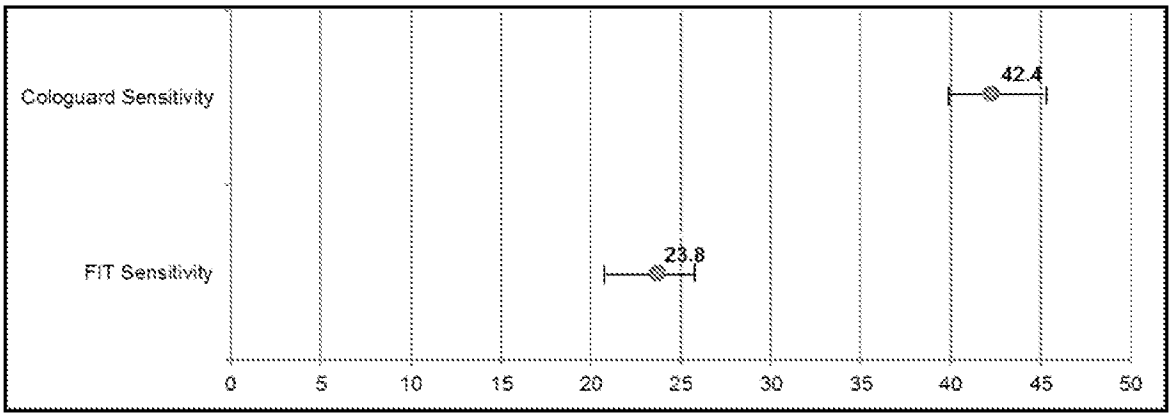
For histopathologically-confirmed AAs, sensitivity was greater for *Cologuard* (42.4%, 321/757) than for FIT (23.8%, 180/757) (**Table 16**). The difference of 18.6% was significantly different from zero (two-sided exact McNemar p value < 0.001), indicating that *Cologuard* is superior to FIT in sensitivity for AA. *Cologuard* identified 170 AA cases that were not called positive by the FIT test, while FIT identified 29 AA cases that were not called positive by *Cologuard* test. Finally, the two-sided 95% CI for the *Cologuard* – FIT difference of 18.6% was 15.3-22.1% (**Figure 2**).

Table 16: Sensitivity Superiority Test – AA Subset (Category 2)

FIT Outcome				McNemar test p-value
Cologuard Outcome	Negative	Positive	Totals	
Negative, n (%)	407 (93.3)	29 (6.7)	436	<0.0001
Positive, n (%)	170 (53.0)	151 (47.0)	321	
Totals	577	180	757	

[1] p-value is from a McNemar paired comparison test of the discordant pairs.

Figure 2: AA Sensitivity



The combined sensitivity for CRC and AA subjects was also analyzed *post hoc*. The sensitivity for CRC/AA was 46.3% (381/822) for *Cologuard* and 27.7% (228/822) for FIT, for a difference of 18.6% (**Table 17**).

Table 17: Sensitivity for Advanced Neoplasia (CRC + AA)

Category	*Cologuard* (N=822)	FIT (N=822)
Category 1 Only	92.3% (60/65)	73.8% (48/65)
Categories 1-2	46.4% (381/822)	27.7% (228/822)

Numerically greater sensitivity for *Cologuard* compared to FIT was observed across all sub-categories of AA. For example, sensitivity for adenoma with carcinoma in situ or high grade dysplasia (Category 2.1) was 69.2% for *Cologuard*, compared to 46.2% for FIT. *Cologuard* identified 43.0% of serrated lesions, whereas FIT sensitivity for these lesions was 5.1%.

For subjects without CRC or AA, the specificity (fraction of subjects called negative) was smaller for *Cologuard* (86.6%, 7936/9167) than for FIT (94.9%, 8695/9167) (**Table 18**). The difference in specificity (–8.3%) was significantly different from zero ($p < 0.0001$). The two-sided 95% confidence interval on the difference was (–9.0%, –7.6%).

For subjects without CRC or AA (categories 3-6), a positive test result is considered a false positive. The false positive fraction is 1 – specificity and was significantly higher for *Cologuard*

(13.4%) than for FIT (5.1%) ($p < 0.0001$). On the other hand, for subjects with CRC or AA, the true positive fraction was higher for *Cologuard* (46.3%) than for FIT (27.7%) (**Table 18**Table).

For subjects without CRC (categories 2-6), the specificity (fraction of subjects called negative) was smaller for *Cologuard* (84.4%, 8372/9924) than for FIT (93.4%, 9272/9924). The difference was –9.1% with two-sided 95% confidence interval (–9.8%, –8.4%). The *Cologuard* specificity for CRC (84.4%) together with its sensitivity for CRC (92.3%) form a complimentary pair spanning the entire study population. By comparison, the FIT specificity for CRC was higher (93.4%) while its sensitivity for CRC was lower (73.8%) than for *Cologuard*.

Table 18: Specificity – Specificity Subset (Categories 3-6)

		FIT Outcome		
	***Cologuard* Outcome**	**Negative**	**Positive**	**Totals**
Categories 3-6	**Negative, n (%)**	7787 (98.1%)	149 (1.9%)	7936
	Positive, n (%)	908 (73.8%)	323 (26.2%)	1231
	Totals	8695	472	9167

Cologuard was also compared with FIT on the Receiver Operating Characteristic curve ("ROC curve"). For tests yielding (but not necessarily reporting) a continuous or ordinal value (measurement or score), a threshold or cut-off may be applied to define test positive and test negative results. The ROC curve is a plot of all possible pairs of sensitivity and 1 – specificity (true and false positive fractions) generated by varying the cut-off over the entire range of observed values.

For CRC, the ROC curves are displayed for *Cologuard* and FIT (**Figure 3**). In the figure, the false positive and true positive fractions associated with cut-offs used by the tests are superimposed. Also displayed in the figure is the area under the ROC curve (AUC) for each test. For *Cologuard* the AUC was 93.0%, indicating that a probability is 93.0% that a randomly chosen CRC subject has a higher underlying *Cologuard* composite score than a randomly chosen non-CRC subject. For FIT this probability was 88.0%.

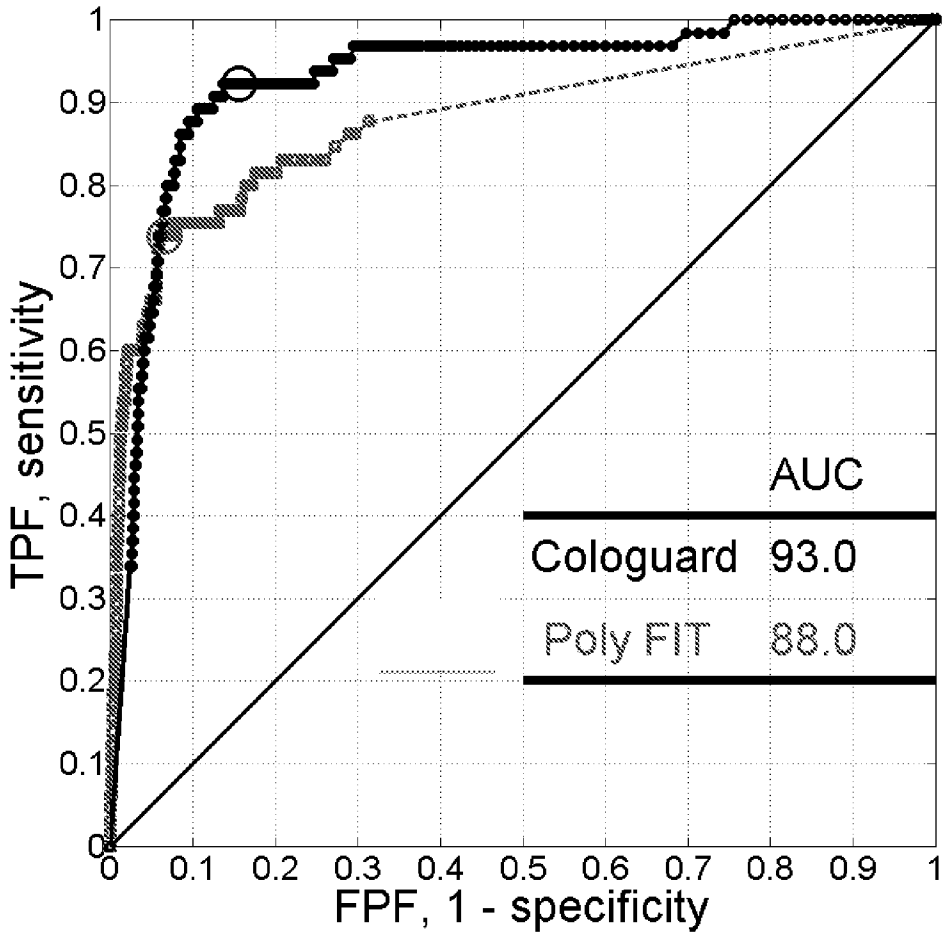


Figure 3: ROC curves for CRC: Cologuard and FIT. The ROC curve plots sensitivity for CRC (Category 1) vs. 1 – specificity for non-CRC (Categories 2-6).

For AN, the ROC curves are displayed for *Cologuard* and FIT (**Figure 4**). For *Cologuard*, the AUC for AN was 73.3%, indicating that a probability is 73.3% that a randomly chosen subject with CRC or AA has a higher underlying *Cologuard* composite score than a randomly chosen subject without CRC or AA. For FIT this probability was 66.7%.

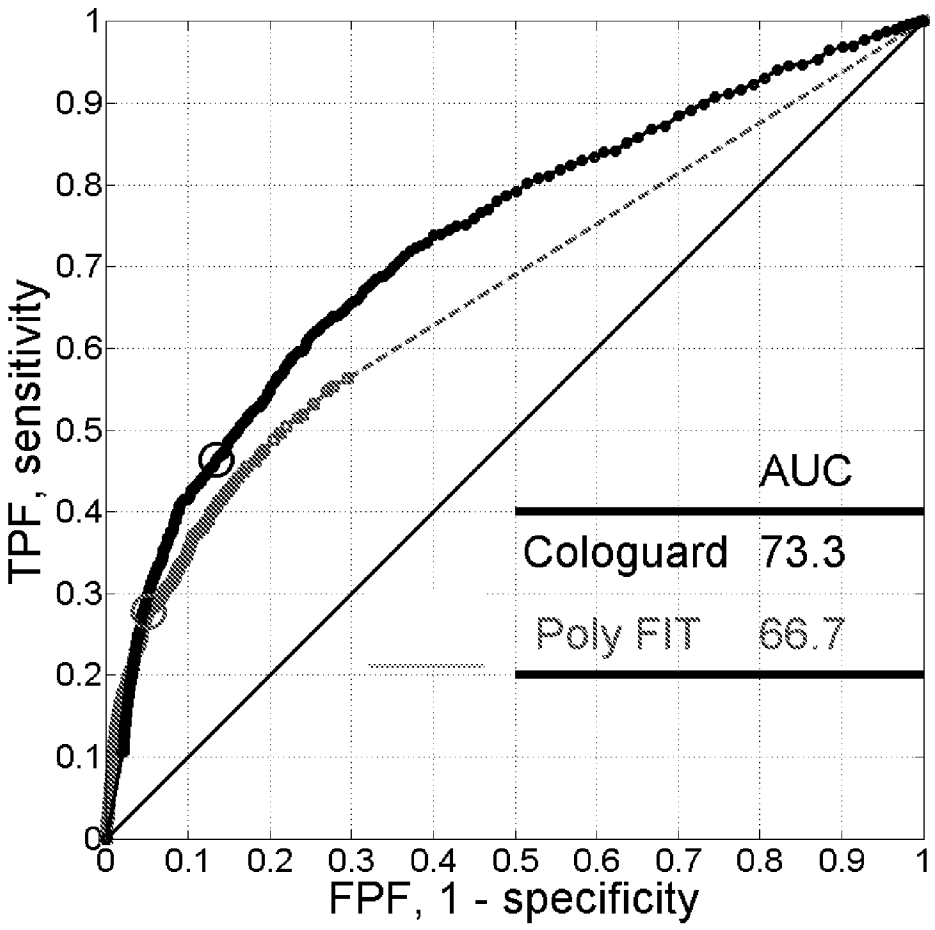


Figure 4*: ROC curves for AN: Cologuard and FIT. The ROC curve plots sensitivity for advanced neoplasia (AN, categories 1-2) vs. 1 – specificity for non-AN (Categories 3-6).*

Additional Effectiveness Analyses

In addition to the sensitivity and specificity for CRC and AA, the positive and negative likelihood ratios for *Cologuard* were calculated from the study data. Results demonstrated a positive likelihood ratio of 5.9 for CRC, indicating that a person with CRC would be 5.9 times more likely to have a positive *Cologuard* result than someone without CRC. The negative likelihood ratio for CRC was 0.09, indicating that someone without CRC is approximately 11 times (1/0.09) more likely to test negative on *Cologuard* compared to someone with CRC.

Results also demonstrated a positive likelihood ratio of 3.2 for AA (**Table 19**), indicating that a person with AA would be 3.2 times more likely to have a positive *Cologuard* results than someone without AA or CRC. The negative likelihood ratio for AA was 0.67, indicating that someone without AA or CRC is approximately 1.5 times (1/0.67) more likely to test negative on *Cologuard* compared to someone with AA.

Table 19: Positive and Negative Likelihood Ratios

	Category 1 (CRC) vs Categories 2-6	Category 2 (AA) vs Categories 3-6
Positive Likelihood Ratio (PLR)		
Sensitivity, %	92.3 (60/65)	42.4 (322/760)
1-Specificity, %	15.6 (1553/9958)	13.4 (1231/9198)
PLR	5.9	3.2
95% Confidence Interval	(5.4, 6.4)	(2.9, 3.5)
Negative Likelihood Ratio (NLR)		
1-Sensitivity, %	7.7 (5/65)	57.6 (438/760)
Specificity, %	84.4 (8405/9958)	86.6 (7967/9198)
NLR	0.09	0.67
95% Confidence Interval	(0.04, 0.21)	(0.63, 0.71)

Analysis was also performed to calculate the positive and negative predictive values ("PPV" and "NPV") for *Cologuard* (**Table 20**). As with any CRC screening test, the PPV is impacted by the very low prevalence of CRC in the general population. The PPV was calculated to be 3.72% (60/1613) for CRC and 19.86% (322/1613) for AA. Meanwhile, the NPV was 94.73%.

Table 20: Positive Predictive Value – Primary Effectiveness Subjects

Cologuard	Category 1 (CRC)	Category 2 (AA)	Categories 3-6
Negative	0.06, 0.02-0.14 (5/8410)	5.2, 4.7- 5.7 (438/8410)	94.7, 94.2-95.2 (7967/8410)
Positive	3.72, 2.85-4.76 (60/1613)	20.0, 18.0-22.0 (322/1613)	76.3, 74.2-78.4 (1231/1613)

*2-Sided 95% CIs

Sub-Group Analyses

The DeeP-C study results were also analyzed according to various demographic characteristics, as well as lesion size and location. Performance goals were not pre-specified for subgroup analysis. No attempt was made to adjust p values for multiple subgroup analyses.

Results by Gender

Sensitivity of *Cologuard* was higher for males than for females, both for CRC and AA. *Cologuard* sensitivity for CRC was 100.0% for males, compared with 83.9% for females (**Table 21**). *Cologuard* sensitivity for AA was 44.7% for males, compared with 39.0% for females.

Table 21: *Cologuard* Sensitivity by Gender (Categories 1 and 2)

Gender, n/N (%)	Category 1 (CRC)	Category 2 (AA)
Male	34/34 (100.0)	201/450 (44.7)
Female	26/31 (83.9)	121/310 (39.0)

[1] Sensitivity calculated as number of positives (CRC or AA) divided by subjects with CRC or AA, respectively.

Meanwhile, specificity of *Cologuard* for subjects with neither CRC nor AA (AN) was very similar for females as compared with males. As shown in **Table 23** below, Specificity for non-AN was 87.3% (4,398/5,037) for females compared with 85.8% (3,569/4,161) for males.

Table 22: *Cologuard* Specificity by Gender

Gender, n/N (%)	**Categories 3-6**[1]
Male	3569/4161 (85.8)
Female	4398/5037 (87.3)

[1] Specificity calculated as number of negatives among subjects without CRC or AA.

Results by Race and Ethnicity

With respect to race, *Cologuard* sensitivity for CRC was higher among White subjects (53/55, 96.4%) than among Black or African-American subjects (5/8, 62.5%). There was only one Asian CRC case in the study (1/1, 100.0%) (**CologuardTable 23)**. However, the results observed in Black or African-American and Asian subjects may well have been driven by the low overall number of cancer cases in that subpopulation. Sensitivity among Hispanic or Latino subjects (8/9, 88.9%) was also high, although again the sample size was small. Sensitivity for AA was similar for White (271/641 42.3%) and Black/African-American (36/85, 42.4%) subjects. Sensitivity was also similar among Hispanic/Latino subjects (23/59, 39.0%). *Cologuard* sensitivity for AA was lower among Asian subjects (4/13, 30.8%) and higher for American Indian or Alaskan Natives (3/4, 75.0%), compared with other groups.

***Cologuard*Table 23: *Cologuard* Sensitivity by Race and Ethnicity, CRC and AA Subsets (Categories 1 and 2)**

Subgroup	Category 1 (CRC)	Category 2 (AA)
Race, n/N (%)		
White	53/55 (96.4)	271/641 (42.3)
Black or African American	5/8 (62.5)	36/85 (42.4)
Asian	1/1 (100.0)	4/13 (30.8)
American Indian or Alaska Native	0/0	3/4 (75.0)
Native Hawaiian or Other Pacific Islander	0/0	0/0
Other	1/1 (100.0)	7/16 (43.8)
Ethnicity, n/N (%)		
Hispanic or Latino	8/9 (88.9)	23/59 (39.0)
Not Hispanic or Latino	52/56 (92.9)	298/700 (42.6)

[1] Sensitivity calculated as number of positives (CRC or AA) divided by subjects with CRC or AA.

Cologuard specificity for subjects without CRC or AA (categories 3-6) varied among race groups (p-value = 0.001) (*Cologuard*Table 27). Specificity was highest for Asian (93.5%, 229/245) and Native Hawaiian/Pacific Islander subjects (91.3%, 21/23) and lowest for American Indian/Alaska Native subjects (75.0%, 24/32).

Table 24: *Cologuard* Specificity by Race and Ethnicity – Primary Effectiveness Subjects

Subgroup	Categories 3-6
Race, n/N (%)	
White	6639/7726 (85.9)
Black or African American	879/978 (89.9)
Asian	229/245 (93.5)
American Indian or Alaska Native	24/32 (75.0)
Native Hawaiian or Other Pacific Islander	21/23 (91.3)
Other	171/189 (90.5)
Ethnicity, n/N (%)	
Hispanic or Latino	837/923 (90.7)
Not Hispanic or Latino	7127/8272 (86.2)

[1] Specificity calculated as number of negatives among subjects without CRC or AA.

Results by Age

Cologuard sensitivity for CRC was consistently high across all age groups (**Table** 25), ranging from 88.9-100.0% for age groups with at least six subjects. Although sensitivity was 75% for subjects of age 60-64, the number of CRC cases was particularly small in this age group (n = 4), and only one CRC case was not detected by *Cologuard*.

Cologuard sensitivity for AA increased from 38.0% for subjects of age < 60 to 46.8% for subjects between age 70-79*Cologuard* (**Table** 25).

Table 25: *Cologuard* Sensitivity by Age

Age, n/N (%)	Category 1 (CRC)	Category 2 (AA)
<60 years	7/7 (100.0)	65/171 (38.0)
60-64 years	3/4 (75.0)	24/57 (42.1)
65-69 years	19/20 (95.0)	125/301 (41.5)
70-74 years	16/18 (88.9)	72/154 (46.8)
75-79 years	6/6 (100.0)	29/62 (46.8)
80-84 years	9/10 (90.0)	7/15 (46.7)

[1] Sensitivity calculated as number of positives (CRC or AA) divided by subjects with CRC or AA.

[2] Two 49-year-old subjects and one 44-year-old subject were included in the analysis population, although they would not be included in the intended use population.

Cologuard specificity for subjects without CRC or AA (categories 3-6) was highest for younger subjects and lowest for older subjects, ranging from 77.8-92.2% (**Table 26**).

Table 26: *Cologuard* Specificity by Age

Age, n/N (%)	Categories 3-6
<60 years	2491/2703 (92.2)
60-64 years	681/765 (89.0)
65-69 years	2871/3352 (85.7)
70-74 years	1292/1566 (82.5)
75-79 years	480/617 (77.8)
80-84 years	152/195 (77.9)

[1] Specificity calculated as number of negatives among subjects without CRC or AA.
[2] Two 49-year-old subjects and one 44-year-old subject were included in the analysis population, although they would not be included in the intended use population.

Results by Lesion Size and Cancer Stage

Cologuard results were evaluated by lesion size, as well as cancer stage (*Cologuard*Table 27). Sensitivity of *Cologuard* increased with lesion size, as would be expected for a stool-based DNA test of this type. The amount of DNA shed from cancerous or pre-cancerous tissue in the colon is generally expected to increase with increased mass or lesion size. *Cologuard*

As shown in the table below, sensitivity was > 90% for most lesion sizes. Sensitivity for CRC was highest for subjects with CRCs ≥ 30 mm (32/34, 94.1%) and lowest for subjects with CRCs 5-9 mm in size (4/5, 80.0%). Sensitivity by cancer stage was generally high and was the highest for subjects with Stage II cancers (21/21, 100.0%) and Stage III cancers (9/10, 90%). Sensitivity of *Cologuard* for AA was higher among subjects with AAs of larger lesion sizes.

***Cologuard*Table 27: *Cologuard* Sensitivity within Lesion Subgroups**

Subgroup	Category 1 (CRC)	Category 2 (AA)
Largest Lesion Size, n/N (%)		
<5 mm	0/0	2/10 (20.0)
5-9 mm	4/5 (80.0)	18/56 (32.1)
10-19 mm	13/14 (92.9)	225/577 (39.0)
20-29 mm	11/12 (91.7)	51/79 (64.6)
>=30 mm	32/34 (94.1)	26/38 (68.4)
Stage, n/N (%)		
I	26/29 (89.7)	N/A
II	21/21 (100.0)	N/A
III	9/10 (90.0)	N/A
IV	3/4 (75.0)	N/A
Unknown*	1/1 (100.0)	N/A

[1] Sensitivity calculated as number of positives (CRC or AA) divided by subjects with CRC or AA.

Specificity of *Cologuard* for subjects without AA or CRC was stratified by lesion size (**Table 28**). Specificity of *Cologuard* for CRC was 86.2% (1,847/2,142), for subjects with CRCs < 5 mm in size, and 79.7% (1,523/1,912) for subjects with CRCs 5-9 mm in size.

Table 28: *Cologuard* Specificity by Lesion Size – Primary Effectiveness Subjects

Largest Lesion Size, n/N (%)	Categories 3-6
<5 mm	1847/2142 (86.2)
5-9 mm	1523/1912 (79.7)
10-19 mm	0/0
20-29 mm	0/0
>=30 mm	0/0

[1] Specificity calculated as number of negatives among subjects without CRC or AA.

Results by Lesion Location

Cologuard sensitivity was also assessed by lesion location (**Table 29**). Sensitivity of *Cologuard* for CRC was 90% or greater, regardless of lesion location. Sensitivity of *Cologuard* for AA was higher among subjects with distal AAs (133/238, 55.9%) and lower among subjects with proximal AAs (143/433, 33.0%).

Table 29: *Cologuard* Sensitivity by Lesion Location

Lesion Location, n/N (%)	Category 1 (CRC)	Category 2 (AA)
Proximal	27/30 (90.0)	143/433 (33.0)
Distal	22/24 (91.7)	133/238 (55.9)
Rectal	11/11 (100.0)	45/88 (51.1)

[1] Sensitivity calculated as number of positives (CRC or AA) divided by subjects with CRC or AA.

Specificity of *Cologuard* for subjects without CRC or AA (categories 3-6) was high, regardless of lesion location. Specificity of *Cologuard* was 83.4% for subjects with proximal CRCs, 82.1% for subjects with distal CRCs, and 84.5% for subjects with rectal CRCs (**Table 30**).

Table 30: *Cologuard* Specificity by Lesion Location – Primary Effectiveness Subjects

Lesion Location, n/N (%)	Categories 3-6
Proximal	1723/2066 (83.4)
Distal	1131/1377 (82.1)
Rectal	517/612 (84.5)

[1] Specificity calculated as number of negatives among subjects without CRC or AA.

Safety Analyses

With respect to safety, due to the design of the study and the nature of the stool collection process, Adverse Effects (AEs) caused by or related to the stool collection procedure were not expected. As a result, events associated with potential errors in use of the collection kit and any product complaints were captured in the safety analyses. There were no cases in which the study investigator believed the product contributed to a serious adverse event, and only 4 adverse events were reported. None of the AEs experienced in the study were deemed serious, all were categorized as “mild” events. None of the events led to the subject discontinuing the study. Additionally, one subject died of unrelated causes prior to undergoing colonoscopy. The subject met all eligibility criteria and successfully collected a stool sample, but did not present for the subsequent colonoscopy.

Abbreviations Used

CRC: Colorectal Cancer
AA: Advanced Adenoma
QuARTS: Quantitative Allele-specific Real-time Target and Signal amplification
KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
ACTB: Beta actin
NDRG4: N-Myc Downstream Regulated Gene 4
BMP3: Bone Morphogenetic Protein 3
Mutation *QuARTS*: Triplex *QuARTS* assay containing wild-type *ACTB* (as a reference gene) and 7 *KRAS* point mutation markers
Methylation *QuARTS*: Triplex *QuARTS* assay containing *ACTB* (as a reference gene), in addition to *NDRG4* and *BMP3* methylation markers

Key Symbols Used

Symbol	Description
IVD	*In vitro* diagnostic medical device
	Consult instructions for use
Σ 480	Contains sufficient reagents for 480 Tests
	Manufacturer
REF	Part number
	Important information for proper operation
NOTE:	Followed by additional information required for the procedure
	Indicates upper and lower temperature limits for storage
	Warning symbol used with specific hazards noted
	Danger, warning, or caution symbol followed by specific precautions

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