DNA Stabilization Is Critical for Maximizing Performance of Fecal DNA-Based Colorectal Cancer Tests

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Abstract: We have developed a multitarget, fecal DNA screening assay that detects the presence of gene-specific mutations and long DNA fragments associated with colorectal cancer (CRC). We continue to investigate methods that may be used to optimize clinical sensitivity. The goals of this investigation are to establish how sample handling conditions affect the stability of DNA in stool, thereby potentially limiting clinical sensitivity, and to determine conditions to ameliorate DNA degradation. A study was run comparing paired sample aliquots. Quantitative PCR data for matched aliquots was used to determine first the effect of sample incubation on total recovery and integrity of DNA, then the effect of stabilization buffer addition to stool on recoverable DNA, and finally the impact of buffer addition on assay sensitivity. Comparison of quantitative PCR data for paired aliquots shows that the amount of recoverable human DNA is negatively affected by storing stool samples (N = 43) at room temperature for ≥36 hours ($P = 0.0018$). However, the addition of stabilization buffer leads to a significant increase in recovery of DNA ($P = 0.010$), compared with samples incubated without buffer. Whereas the DNA Integrity Assay (DIA) is found to be sensitive to DNA degradation (sensitivity was reduced by 82%; $P = 0.0002$), point mutation marker sensitivity is more refractory. Overall, DNA can be stabilized by addition of buffer to the sample, leading to increased assay sensitivity.

Key Words: colorectal cancer, multitarget assay, DNA integrity, cancer screening, stool DNA

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Colorectal cancer (CRC) is the fourth most prevalent cancer in the United States and is the second leading cause of cancer deaths.[1] More than 90% of colorectal cancer cases could be cured if detected in its earliest stages.[2] Current colorectal cancer screening guidelines include a variety of options including fecal occult blood test (FOBT), flexible sigmoidoscopy, double-contrast barium enema, and colonoscopy.[1–3] Whereas being the most sensitive,[4] the financial costs, manpower requirements, and potential complications associated with colonoscopy present formidable obstacles to its implementation for large-scale, nationwide CRC screening.[5] The other methods are less sensitive and are either invasive, or in the case of FOBT, depend upon a nonspecific, indirect assessment of blood in fecal matter. Fecal DNA methods have been developed that are noninvasive and present continued opportunity for improvement as new molecular markers associated with CRC are identified and as new DNA detection technologies are developed. Results of several targeted studies to assess sensitivity and specificity of fecal DNA tests have been previously reported,[6–9] with sensitivities ranging from 52% to 91% and specificities of 93% to 98%. Although these studies offer a confirmation of the potential benefits of fecal DNA screening protocols, it is known that several variables can affect test performance. Markers must be chosen that yield an acceptable clinical sensitivity for the intended application (ie, screening average-risk individuals for sporadic disease). The fecal DNA assay is based on a combination of a panel of point mutations in *APC*, *p53*, and *Kras* genes, as well as a microsatellite instability (MSI) marker, BAT-26, and a marker for long DNA fragments, DNA integrity assay (DIA). Additionally, mutation detection methods must be chosen that offer sufficient analytical sensitivity because the human DNA recovered from stool is highly heterogeneous. Normal cells are sloughed into the colonic lumen along with the mutant cells. Therefore, analytical methods must be chosen that can detect as little as 1% mutant DNA in the presence of excess wild-type DNA. Also, sample prep methodologies must be chosen that allow for maximum recovery of human DNA from samples. Most of the DNA recovered from stool is bacterial in origin, with the human DNA component representing only a small minority. Purification methodologies must be able to efficiently select for the rare human component, and because the mutant copies (when they exist) represent only a small percentage of the total human DNA from stool, it is important to maximize the recovery of human DNA to maximize the probability of amplifying mutant copies in the PCR reactions. Development of a new affinity gel electrophoresis method that meets these needs has recently been described.[10] Lastly, it is imperative to preserve the DNA in stool, such that it does not degrade during sample handling. A common method to ensure that DNA remains stable is to freeze stool samples as quickly as possible after collection or to receive samples in centralized testing labs as quickly as possible. However, to provide the option of decentralized sample analysis and still retain maximum sample integrity, it is desirable to develop a more robust and standardized sample-handling method.

A multicenter study was recently completed to evaluate the sensitivity of a multitarget fecal DNA assay relative to FOBT in an average-risk population.[9] The study had 31 cancers, confirmed through colonoscopy, by screening approximately 5,000 patients, and the majority of cancers were found to represent early-stage disease. Even though the study demonstrated a 4-fold greater sensitivity than FOBT, the fecal

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DNA assay was expected to detect a greater proportion of the cancers. The sensitivity contributed by the point mutation panel of markers was found to be consistent with previous studies,[6–8] but the DIA portion of the test contributed significantly lower sensitivity to the overall assay than what had been seen previously, raising the question of how sample handling may affect DNA stability, in general, and the sensitivity of the different parts of the multitarget assay, more specifically.

With the possibility that DNA degradation might lead to loss of marker sensitivity and overall assay performance, methods of making sample collection and handling more robust were considered. Here we present experimental results that not only demonstrate how sample handling can affect DNA stability but also how degradation can be ameliorated by addition of buffer to stool samples shortly after collection.

MATERIALS AND METHODS

Sample Collection and Incubation

A total of 43 samples were collected from known CRC patients as well as patients without cancer by a separate organization (Indivumed GmbH, Hamburg, Germany) that also managed all patient informed consent and compliance with human subject guidelines. All stool samples were frozen within 1 hour of defecation and shipped to EXACT Sciences on dry ice (−78°C). Once received, samples were subjected to prescribed room temperature incubation times as described later. Prior to the start of the incubation time course, stool samples were thawed and 1 aliquot was processed to recover DNA immediately (t_0). The DNA from the t_0 aliquot for all samples was analyzed and served as an incubation control. The remainder of the stool was left to incubate at room temperature. At prescribed time points, aliquots were removed from the stool and human DNA was recovered and analyzed in similar manner to the controls.

All aliquots were standardized by weight (30 g). Experiments were designed to measure the effect of incubation time on DNA integrity, as well as the quantity of recoverable DNA. The experiments also included an addition of stabilization buffer to stool aliquots. Stabilization buffer consisted of 0.5 mol/L Tris, 0.15 mol/L EDTA, and 10 mmol/L NaCl (pH 9.0). In these experiments, aliquots were stored at room temperature for 36 or 48 hours, with and without buffer added. In the case of buffer addition, the buffer was simply added to the stool aliquot in a plastic container with a lid, but no effort was made to homogenize the sample. At the prescribed time period, the aliquots with and without buffer were processed to recover human DNA and then analyzed by the DIA assay. An additional set of experiments was conducted to study the effect of incubation time on specific gene mutations. In this experiment, 1 set of samples (6 samples) was incubated for 36, 48, or 72 hours without any stabilization buffer added. Another set of samples (5 samples) was incubated for 36 or 48 hours, with and without buffer added. After the prescribed incubation time, all samples were processed to recover and purify human DNA and analyze the DNA for gene mutations as described later.

Recovery of DNA From Stool

The sample preparation methodology used to recover DNA from stool was previously reported.[6,10] Stool aliquots were weighed and combined with Exact buffer A (1:7 (w/v) ratio) and homogenized on an Exactor (Exact Sciences). After homogenization, a 4-g stool equivalent (~32 mL) of each sample was centrifuged to remove all particulate matter. The supernatants were then treated with 20 μL TE buffer (0.01 mol/L Tris [pH 7.4] and 0.001 mol/L EDTA) containing RNase A (2.5 mg/mL) and incubated at 37°C for 1 hour. Total nucleic acid was then precipitated (first adding 1/10 volume 3 mol/L NaAc, then an equal volume of isopropanol). Genomic DNA was pelleted by centrifugation, the supernatant removed, and the DNA resuspended in TE.

Human DNA Purification

Target human DNA fragments were purified from total nucleic acid preparations using a newly developed DNA affinity electrophoresis purification methodology. This method has recently been described in detail.[10] In brief, human DNA can be separated from the excess bacterial DNA by hybridization of the target sequences to complementary, covalently bound oligonucleotide capture probes in acrylamide gel membranes. Crude human DNA preparations (2,400 μL) were mixed with 960 μL formamide (Sigma), 385 μL 10× TBE, and filtered through a 0.8-μm syringe filter (Nalgene, Rochester, NY) and then denatured (heated at 95°C for 10 minutes, then cooled in ice for 5 minutes). The sample mix was loaded on top of the capture membrane, and electrodes above and below the capture layer were applied. Samples were electrophoresed (15 V, 16 hours) using TBE in the reservoirs above and below the capture layer. After electrophoretic capture, the remaining solution was removed from the tubes, and the tube array was separated from the capture plate. The capture membranes were then washed and 40 μL of 100 mmol/L NaOH was added to the top of the capture membrane and incubated for 15 minutes. The capture plate was placed on top of a custom molded 48-well DNA collection plate and centrifuged briefly (1,900 × g) to recover the eluted DNA. Then, 8 μL of neutralization buffer (500 mmol/L HCL + 0.1× TE) was added to each well of the collection plate and mixed.

Quantification of Recovered Human DNA by TaqMan Analysis

TaqMan analysis was performed on an I-Cycler (BioRad) with primers against a 200-bp region of the *APC* gene. A probe labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) was used to detect PCR product. Amplification reactions consisted of captured human stool DNA mixed with 10× PCR buffer, LATaq enzyme (Takara), 1× PCR primers (5 μmol/L), and 1× TaqMan probe (2 μmol/L; Biosearch Technologies). We used 5 μL of captured DNA in the PCR reactions. TaqMan reactions were performed with the same program as described below (DIA).

Sequence-Specific Amplification

Polymerase chain reaction (PCR) amplifications (50 μL) were performed on MJ Research Tetrad Cyclers (Watertown, MA) using 10 μL of purified DNA, 10× PCR buffer

(Takara Bio Inc, Madison, WI), 0.2 mmol/L dNTPs (Promega, Madison, WI), 0.5 μmol/L sequence-specific primers (Midland Certified Reagent Co, Midland, TX), and 2.5 U LATaq DNA polymerase (Takara). All amplification reactions were performed under identical thermocycler conditions: 94°C for 5 minutes and 40 cycles consisting of 94°C (1 minutes.), 60°C (1 minute), and 72°C (1 minute), with a final extension of 5 minutes at 72°C. Thirteen separate PCR reactions were run per sample. For analysis of each of the PCR products, 8 μL of each amplification reaction was loaded and electrophoresed on a 4% ethidium bromide–stained NuSieve 3:1 agarose gel (FMC, Rockland, ME) and visualized with a Stratagene EagleEye II (Stratagene, La Jolla, CA) still image system. All oligonucleotide sequences (capture probes, PCR primers, and TaqMan probes) are available upon request.

The multitarget assay was designed to have 13 separate PCR reactions in the multiple mutation (MuMu) panel and 12 PCR reactions in the DIA portion of the assay.

Mutation Panel Analysis

The presence or absence of point mutations or Bat-26–associated deletions was determined by using modified solid-phase single-base extension (SBE) reactions. Point mutation targets included codons K12p1, K12p2, and K13p2 on the K-*ras* gene; codons 876, 1306, 1309, 1312, 1367p1, 1378p1, 1379, 1450p1, 1465 and 1554 on the *APC* gene; and codons 175p2, 245p1, 245p2, 248p1, 248p2, 273p1, 273p2, and 282p1 on the *p53* gene. Including the Bat-26 deletion marker, the panel consisted of 22 markers in total. For all gene targets, separate wild-type and mutant specific reactions were performed. Details of the reactions and analysis using capillary electrophoresis have been previously described.[10]

DIA

The DIA assay has been previously described in detail.[11] More recently, this assay has been converted to a real-time PCR methodology (unpublished data). Three unique PCR reactions (in duplicate) per loci were run on I-Cycler instruments (BioRad, Hercules, CA). The strategy was to capture locus-specific segments and perform small (~100 bp) PCR amplifications remote from the capture site as an indicator of DNA length. DNA fragments for integrity analysis were amplified from 4 different loci: 17p13, 5q21, HRMT1L1, and LOC91199. PCR primer sets and associated TaqMan probe for each loci of interest are "walked" down the sequence, thereby interrogating for the presence and quantity of increasing length of DNA of approximately 1,300 bp, 1,800 bp, and 2,400 bp fragments of captured DNA. Purified DNA template (5 μL) was mixed with 5 μL 10× PCR buffer (Takara), 10 μL dNTPs (2 mmol/L, Promega), 0.25 μL LATaq (5 U/μL, Takara), 24.75 μL molecular biology grade water (Sigma), and 5 μL of a mix of PCR primers (5 μmol/L, Midland) and TaqMan dual-labeled probes (2 μmol/L, Biosearch Technologies). The I-Cycler was programed as follows: 94°C for 5 minutes and then 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. Genomic standards, prepared as 20, 100, 500, 2,500, and 12,500 GE/5 μL, were prepared and used to generate a standard curve.

DIA Data Analysis

Threshold genome equivalents (GE) values were determined for each of 12 PCR reactions (corresponding to the 1.3kb, 1.8kb, and 2.4kb fragments across the 4 genomic loci) using a previously determined set of cancers and normals. We then applied a requirement that at least 4 of the 12 PCR reactions are above the individual PCR thresholds to prospectively determine cancers.

Statistical Methods

The impact of sample incubation on DNA recovery and the impact of stabilization buffer on DNA recovery were both assessed using quantitative PCR analysis. The data for both comparisons were subjected to a paired-sample *t* test using GraphPad QuickCalcs software (GraphPad Software, Inc, San Diego, CA). The effect of sample incubation on the integrity of recoverable DNA was analyzed using DIA cutoffs. Resulting DIA scores after incubation were analyzed by a Fisher exact test compared with controls that had been analyzed at t_0, using GraphPad QuickCalcs.

RESULTS

Effect of Sample-Handling Conditions on DIA

DIA is a measure of long DNA which has been shown to be an independent and effective marker of CRC.[11] In these experiments, the effect of sample-handling conditions on recoverable DNA is assessed using DIA. Moreover, the results of the DIA analysis are a direct indication of how this marker is impacted under the prescribed conditions and likewise how CRC detection sensitivity is affected. A total of 38 samples were analyzed by DIA. Twenty-seven were DIA negative at t_0, and aliquots were incubated with and without stabilization buffer. There was no significant amount of long DNA present in these samples to judge the effect of incubation conditions on DNA stability. However, impact of sample-handling conditions was assessed by total recoverable DNA (ie, 200 bp results). A sampling of results from this group of specimens is shown in Table 1. A DNA recovery score was calculated by averaging the results for the 4 separate loci (D, E, X, and Y) for each sample. Without any buffer added, the majority of samples (18/27, 67%) stored at room temperature (≥36 hours) yielded less than 50% of the DNA recovered at t_0. The remaining 9 samples (33%) had mild loss of recovery, yielding equivalent DNA, or less than a 50% loss, upon room temperature incubation. When aliquots were incubated with buffer, 81% (22/27) of the DIA-negative samples were preserved (samples GP30, GP33, and GP96, in Table 1, are shown as examples). In the remaining 5 DIA-negative samples, the addition of stabilization buffer did not offer any significant advantage in recoverable DNA, compared with samples stored without buffer (see for example, LSP20-21 in Table 1).

Of the 38 samples analyzed by DIA, 11 were found to be positive at t_0. Table 2 shows the detected copy numbers of each DIA marker for these samples at t_0 and extended incubation times, with and without stabilization buffer added. The DIA score (number of positive markers per sample) is also indicated. When no stabilization buffer is added, DNA is

TABLE 1. Quantification of Recoverable Human DNA from Selected DIA-Negative Samples Incubated With and Without Stabilization Buffer

Sample ID	Incubation Conditions	D200 (GE/10 μL)	E200 (GE/10 μL)	X200 (GE/10 μL)	Y200 (GE/10 μL)
GP30	Fresh	247	196	418	129
	36 h no buffer	50	27	76	0
	36 h with buffer	219	216	390	172
GP96	Fresh	129	1,120	604	769
	36 h no buffer	63	130	100	100
	36 h with buffer	34	1,270	1,140	1,290
GP33	Fresh	3,690	13,200	23,300	0
	36 h no buffer	61	136	422	103
	36 h with buffer	2,220	4,640	3,860	2
LSP20-21	Fresh	1,140	807	1,500	337
	36 h no buffer	219	588	421	285
	36 h with buffer	542	363	174	591

significantly degraded in 9 of the 11 originally DIA-positive stool samples when stored for ≥36 hours at room temperature. These would therefore be graded as DIA negative after room temperature incubation, resulting in an 82% loss in sensitivity ($P = 0.002$) for DIA. However, for these same samples, addition of stabilization buffer prior to room temperature incubation yields significantly higher DNA copy number, such that all of the samples (11/11) would remain DIA positive, even after room-temperature incubation. Two of the samples (GP-031 and GP-079) yielded high quantities of long DNA fragments even upon extended room temperature incubation without any added stabilization buffer.

Effect of Sample Handling Conditions on Gene Mutation Markers

It has previously been shown that DNA recovered from stool can be interrogated for specific mutations known to be associated with CRC.[6–8] In the experiments described earlier, it was shown that upon room temperature incubation of stool samples long fragments of DNA are degraded, significantly diminishing the usefulness of the DIA markers. Further it was shown that human DNA yield is reduced introducing the question of whether or not sufficient amount of DNA template molecules remain for point mutation analysis in known CRC associated genes (eg, *Kras*, *APC*, and *p53*). Stool samples from 11 confirmed CRC patients were collected and shown to contain 1 or more point mutations, as summarized in Table 3.

The amount of DNA recovered from aliquots stored under the different conditions is based on quantification of a 200-bp DNA fragment (Table 3). Although the amount of recoverable DNA varies widely from sample to sample, at t_0 the average recovery was 14,891 copies/10 μL, whereas after room temperature incubation, the average recovery was 1,955. Without buffer added to the samples, between 65% to 98% of the DNA was no longer recoverable (excluding sample GP-031), after incubation of samples at room temperature, compared with t_0. The one exception, sample GP-031, maintained high DNA yield even without addition of stabilization buffer. Samples incubated with stabilization buffer maintained human DNA yields similar to the t_0 samples (Table 3).

Aliquots from all samples were analyzed for mutations initially (t_0), and additional aliquots were analyzed after room temperature incubation. Aliquots from the first 6 stool samples (Table 3) were simply stored at room temperature with no buffer added, whereas aliquots from the next set of 5 samples were stored with and without the addition of stabilization buffer. Mutations were reproducibly detected in 10 of the 11 samples. Sample GP-105 was originally shown to contain an *APC* mutation (at codon 1554), and after incubation of an aliquot for 48 hours without buffer, the mutation was no longer detectable even with repeated analysis. However, when GP-105 was incubated in the presence of stabilization buffer, the originally identified mutation was detected. In addition, as we observed within the DIA marker experiments, human DNA recovery for all samples remained high when incubated in the presence of stabilization buffer, and DNA recovered from these aliquots also maintained detectable mutations.

DISCUSSION

The ability to recover human DNA from stool samples and identify mutations associated with colorectal cancer has been shown by several groups over the last decade. Sidransky et al[12] first reported interrogating K-*ras* mutations associated with sporadic CRC in stool DNA in 1992. Subsequent reports also involved interrogation of single genetic targets.[13–17] More recently, assays with multiple markers have been developed[18,19] to yield increased assay sensitivity in light of the genetic heterogeneity of sporadic CRC cases. Furthermore, an assay for long DNA recovered from stool has been developed and shown to be associated with CRC with high specificity.[11] The DIA itself was shown to detect 57% of cancers in one study[11] and has been shown to detect from 37%[8] to 67%[6] of cancers when incorporated in a multitarget assay. The multitarget tests have the potential to be used in population-based screening applications. However, in all cases, one of the central challenges is to preserve the integrity of human DNA in the hostile stool environment, particularly during sample transport, to recover, amplify, and interrogate the DNA for known cancer-related abnormalities. Nucleases that are active in stool have the potential to rapidly degrade DNA, including the minor human

TABLE 2. Results of DNA Integrity Assay for DIA-positive Samples Incubated at Room Temperature for Prescribed Times, With and Without Stabilization Buffer. DIA Scores (A) and Total Recoverable DNA (B) are Shown for 11 Samples Found to be DIA-positive at t_0

(A)

		DIA-D			DIA-E			DIA-X			DIA-Y			
Sample	**Time Point**	**1.3KB**	**1.8KB**	**2.4KB**	**1.3KB**	**1.8KB**	**2.4KB**	**1.3KB**	**1.8KB**	**2.4KB**	**1.3KB**	**1.8KB**	**2.4KB**	**LcDIA Score**
GP32	0 HR	15	0	0	126	34	37	15	15	18	2	1	4	3
	36 HR NB	0	18	14	38	20	14	1	0	1	9	21	6	0
	36 HR B	55	52	27	69	15	19	16	21	26	13	14	15	7
GP29	0 HR	394	522	150	1490	734	343	141	98	118	1	3	2	9
	24 HR NB	0	0	0	0	0	0	1	0	0	11	8	17	1
	24 HR B	812	1490	603	2030	1090	710	202	476	198	150	236	247	12
	36 HR NB	0	0	0	1230	0	0	2	1	2	5	12	8	1
	36 HR B	10	26	21	1170	1050	768	105	52	53	1	2	3	6
GP34	0 HR	1180	840	318	4890	3160	2180	247	387	299	1	1	2	9
	24 HR NB	10	13	0	13	11	0	5	4	6	6	7	9	0
	24 HR B	6170	3890	2230	11800	8960	7070	18	12	12	2450	1620	4930	10
	36 HR NB	0	0	0	17	6	0	2	0	2	2	2	2	0
	36 HR B	1250	2090	959	7650	3090	2760	366	516	353	859	577	1720	12
GP38	0 HR	735	598	202	3930	2300	3000	2	1	1	114	103	243	9
	36 HR NB	0	0	0	22	9	0	1	2	1	0	0	0	0
	36 HR B	88	81	35	426	369	310	4	6	5	39	19	26	7
GP31	0 HR	200	223	134	992	493	250	230	125	108	47	36	44	11
	36 HR NB	274	441	205	1320	573	241	66	45	111	52	79	41	11
	36 HR B	129	140	56	257	116	41	30	24	22	ND	ND	ND	12
107	0 HR	672	1310	236	1020	813	471	1770	137	138	1060	908	605	12
	48 HR	0	0	0	5	4	0	1	1	4	3	2	220	1
	48 HR, Buffer	567	1120	294	647	541	308	1110	101	89	615	778	576	12
117	0 HR	170	519	78	320	130	108	255	24	15	453	357	312	12
	48 HR	0	0	0	0	0	0	1	1	2	0	0	0	0
	48 HR, Buffer	1170	7590	755	3840	3000	2810	4490	3790	4860	1930	1640	1540	12
97	0 HR	36	202	10	78	33	10	15	5	4	79	123	43	5
	48 HR	0	0	0	0	3	0	2	0	3	1	1	0	0
	48 HR, Buffer	50	84	22	116	63	26	45	16	34	141	121	123	9
105	0 HR	71	233	47	258	181	83	123	18	11	167	120	315	11
	48 HR	0	61	0	0	2	0	1	0	1	1	2	0	1
	48 HR, Buffer	29	118	9	81	50	33	18	2	2	67	44	46	7
90	0 HR	469	1560	167	86	26	96	942	65	91	1420	890	1380	11
	48 HR	0	0	0	4	6	0	1	0	1	4	2	8	0
	48 HR, Buffer	1320	8290	398	13500	4870	4600	2490	183	67	2420	1790	6270	12
GP79	0 HR	250	21	21	1250	107	124	44	62	52	392	728	0	9
	48 HR	72	20	11	528	53	64	21	27	22	192	370	676	8
	48 HR, Buffer	128	70	24	446	89	87	35	51	36	241	663	0	10

(B)

		DIA-D	DIA-E	DIA-X	DIA-Y
Sample	**Time Point**	**200**	**200**	**200**	**200**
GP32	0 HR	4690	3830	14000	4040
	36 HR NB	1160	752	309	1490
	36 HR B	14700	2460	7720	15000
GP29	0 HR	2120	3340	5560	9
	24 HR NB	92	161	505	140
	24 HR B	2380	3720	6230	2890
	36 HR NB	131	324	1010	8
	36 HR B	58	2770	2750	11
GP34	0 HR	15800	34600	36100	293
	24 HR NB	672	951	3600	571

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