

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 $\times$ 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 ($1,900 \times g$) to recover the eluted DNA. Then, 8 μL of neutralization buffer (500 mmol/L HCL + 0.1 $\times$ 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 $\times$ PCR buffer, LATaq enzyme (Takara), 1 $\times$ PCR primers (5 $\mu\text{mol/L}$), and 1 $\times$ TaqMan probe (2 $\mu\text{mol/L}$; 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 $\times$ PCR buffer

(Takara Bio Inc, Madison, WI), 0.2 mmol/L dNTPs (Promega, Madison, WI), 0.5 μ mol/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 μ L 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 μ L) was mixed with 5 μ L 10 $\times$ PCR buffer (Takara), 10 μ L dNTPs (2 mmol/L, Promega), 0.25 μ L LATaq (5 U/ μ L, Takara), 24.75 μ L molecular biology grade water (Sigma), and 5 μ L of a mix of PCR primers (5 μ mol/L, Midland) and TaqMan dual-labeled probes (2 μ mol/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 μ L, 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 μ L)	E200 (GE/10 μ L)	X200 (GE/10 μ L)	Y200 (GE/10 μ L)
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 μ L, 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 al¹² 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 study¹¹ 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)														
Sample	Time Point	DIA-D			DIA-E			DIA-X			DIA-Y			LcDIA Score
		1.3KB	1.8KB	2.4KB	1.3KB	1.8KB	2.4KB	1.3KB	1.8KB	2.4KB	1.3KB	1.8KB	2.4KB	
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)														
Sample	Time Point	DIA-D			DIA-E			DIA-X			DIA-Y			
		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			

(continued on next page)

TABLE 2. (continued) 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

		(B)			
		DIA-D	DIA-E	DIA-X	DIA-Y
Sample	Time Point	200	200	200	200
GP38	24 HR B	68200	72700	193	61400
	36 HR NB	279	471	517	77
	36 HR B	29400	31600	27400	24000
	0 HR	35500	46400	28	16700
	36 HR NB	103	375	284	47
GP31	36 HR B	6320	10300	7510	7930
	0 HR	4100	3290	5960	2510
	36 HR NB	7220	6390	3060	3230
107	36 HR B	3400	1080	4990	ND
	0HR	17900	6890	14900	5970
	48 HR NB	23	21	15	23
117	48 HR B	3600	1860	3710	1790
	0HR	6230	2620	3340	2830
	48 HR NB	4	4	3	3
97	48 HR B	12100	7120	9020	3770
	0HR	2790	2100	2260	2090
	48 HR NB	16	12	33	22
105	48 HR B	1400	1180	1920	1690
	0HR	1690	1420	1660	1320
	48 HR NB	24	30	13	29
90	48 HR B	379	549	469	626
	0HR	36000	1070	15900	9520
	48 HR NB	175	244	54	198
GP79	48 HR B	72900	30900	22700	10500
	0 HR	4470	20400	12200	13200
	48 HR NB	3680	14600	9790	6370
	48 HR B	3980	14100	8510	8190

■ DIA-positive marker; □ DIA-positive sample.

DNA component, and measures must be taken to minimize their negative impact. In this study, we investigate the influence of adding EDTA-containing buffers to complex samples, such as stool, to preserve DNA.

One of the characterizing features of cancer is loss of normal cell regulation, including escape from apoptosis. It has been proposed that CRC may lead to enhanced exfoliation of colonocytes.²⁰ Further, several groups^{6,13,21,22} have reported an association of increased recovery of DNA from stools of patients found to have colorectal cancer. Similarly, detection of cell-free DNA in plasma and serum has received much attention as a potential marker for detection and monitoring of cancers. There are many accounts of analysis of circulating DNA for cancer-related genetic mutations.²³ Also, associations of increased quantity^{24–27} and molecular size^{28,29} of cell-free DNA detected in plasma and serum with common cancers including CRC have been reported. The DIA assay was developed to interrogate for the presence of long DNA,¹¹ as an extension to the detection of DNA-quantity in bodily fluids, to increase assay sensitivity. It has also been shown that the DIA assay adds increased sensitivity to an assay based on a panel of mutations in known CRC-associated genes,^{6,8} indicating it is

an important marker. Our results indicate that without taking steps to preserve the integrity of DNA in stool samples, the majority of DIA-positive samples (ie, 82% [9/11] in this study) will be degraded leading to false-negative results. Although the duration that clinical samples may be subjected to unfavorable temperatures may vary in practice during sample transport, we believe that there is a need to develop a standardized method for sample collection and handling to preserve this important marker. Addition of stabilization buffer to stool samples prior to transport presents an easily implemented solution that appears to be highly effective. We observed some variation in copy numbers of long DNA fragments comparing samples incubated with buffer versus samples analyzed fresh, but these differences were inconsequential with respect to DIA scores. All of the samples (11/11, 100%) that were DIA positive at t_0 remained positive after incubating at room temperature for ≥ 36 hours when buffer was added to the stool sample, whereas without buffer addition only 18% of the samples (2/11) remained positive.

A total of 43 samples were analyzed in this study. Thirty-eight were evaluated by the DIA assay, 11 of which were found to be DIA positive, as discussed earlier. Eleven samples were

TABLE 3. Gene Mutation Analysis Results for Stool Aliquots Incubated At Room Temperature

Sample	Conditions	DNA Quant.	Marker	SBE Threshold	SBE Signal	P/N
GP-003	t_0	2,180	<i>Kras, k12p2</i>	0.400	0.445	POS
	72 h	351			1.504	POS
	t_0		<i>p53, 273p1</i>	0.200	0.999	POS
	72 h				1.705	POS
GP-023	t_0	59,800	<i>Kras, k12p2</i>	0.400	1.071	POS
	72 h	3,250			2.423	POS
GP-024	t_0	509	<i>BAT-26</i>	0.050	0.093	POS
	48 h	119			0.380	POS
	72 h	217			0.092	POS
GP-025	t_0	788	<i>p53, 248p2</i>	1.000	6.648	POS
	48 h	121			1.206	POS
	72 h	125			3.697	POS
GP-026	t_0	458	<i>p53, 175p2</i>	0.400	1.459	POS
	48 h	160			1.914	POS
	72 h	185			0.449	POS
GP-034	t_0	72,300	<i>p53, 248p2</i>	1.000	1.505	POS
	36 h	1,180			4.834	POS
GP-029	t_0	10,700	<i>p53, 175p2</i>	0.400	1.229	POS
	36 h	1,110			1.721	POS
	36 h, Buffer	11,000			1.793	POS
GP-030	t_0	501	<i>Kras, k13p2</i>	0.400	1.814	POS
	36 h	135			3.650	POS
	36 h, Buffer	338			2.593	POS
GP-031	t_0	10,300	<i>APC, 1309</i>	0.055	0.219	POS
	36 h	13,500			0.090	POS
	36 h, Buffer	4,550			0.210	POS
GP-105	t_0	1,540	<i>APC, 1554</i>	0.250	1.210	POS
	48 h	78			0.049	NEG
	48 h, Buffer	9,140			2.392	POS
GP-121	t_0	4,720	<i>p53, 175p2</i>	0.400	2.699	POS
	48 h	1,500			1.601	POS
	48 h, Buffer	1,040			2.329	POS

Sample handling conditions and analysis frequency is described in the materials and methods section. All aliquots were analyzed for total recoverable DNA (DNA Quant) as well as for the presence of any of 22 mutations in *K-ras*, *APC*, and *p53* genes, as well as BAT-26.

analyzed by the mutation panel assay of 22 point mutations. Six of the samples were analyzed by both DIA and the mutation panel. Analyzing all of the samples together ($N = 43$), statistical analysis (1-tailed paired t test) shows a significant decrease ($P = .0018$) in recovered DNA for aliquots that had been incubated at room temperature without buffer (control = t_0). The addition of buffer to samples prior to room temperature incubation was found to yield a significant increase in DNA recovery, relative to the matched aliquots with no buffer added ($P = .010$). Addition of stabilization buffer prior to room temperature incubation preserves DIA positives for all samples (11/11), whereas without buffer addition only 2 of 11 samples remained DIA positive ($P = .0002$, Fisher exact test).

There was no apparent benefit in recoverable DNA, with buffer addition, for 5 samples which were all originally DIA negative (whereas with DIA-positive samples 100% [11/11] of the samples benefited). In general, we observed that DIA-negative samples contained less recoverable human DNA

compared with DIA-positive samples, which is consistent with previous observations of increased DNA recovery from cancer patients.^{6,24–27} The median recovery for the DIA-negative samples was 452 GEs and 8,410 for the DIA-positive samples ($P < .0001$). Overall, this suggests that DIA-positive samples have almost 20-fold more human DNA recoverable from stool. The 5 samples that did not demonstrate any benefit from buffer addition all had low recovery of DNA at t_0 . We have not investigated why these samples are not preserved by addition of buffer to stool but observe that they may represent a small percentage of the normal population where even the addition of EDTA cannot overcome the action of nucleases when only small amounts of human DNA are released into the lumen of the colon.

Although the DIA markers are sensitive to room temperature incubation, the mutation panel markers are shown to be more robust. Despite a large loss of DNA for samples stored at room temperature, the original mutations could be

confirmed in 10 of 11 samples (91%). As an example, sample GP-003 yielded 2180 GE of human DNA at t_0 and was originally found to be positive for a *Kras* (k12p2) and a *p53* (273p1) marker. Upon room temperature incubation of a stool aliquot for 72 hours, only 351 GE (equivalent to an 84% loss) of amplifiable DNA was recovered, yet the sample remained positive for both markers. Only 1 sample, GP-105, exhibited a loss in mutation detection (for APC 1554) and was also the only sample that yielded less than 100 copies of DNA after room temperature incubation without buffer. However, with stabilization buffer added, over 9,000 copies were recovered even after 48 hours of incubation, and the sample remained positive for the APC mutation. Six samples (GP-029, GP-030, GP-031, GP-034, GP-105, and GP121) were analyzed by both DIA as well as the mutation panel. All 6 were initially positive for at least one of the point mutations (Table 3), 4 were also DIA positive, and 2 were DIA negative (Table 2). Although there is clearly overlap of the point mutation and DIA markers for some samples, we do not know if there is any biologic association between the 2 sets of markers.

Results from these experiments indicate that DIA is quite sensitive to sample incubation conditions and DNA degradation, whereas the mutation panel is more refractory. This is likely due to the fact that DIA is based on a direct assessment of long-DNA copy number and incubation of samples at room temperature for 36 hours or more degrades DNA to the point that remaining long fragments fall below the assay cutoff. The point mutation assay, on the other hand, simply requires that the analytical detection sensitivity is sufficient for rare mutant copies in the presence of excess wild-type sequences (ie, 1% mutant detection). Total human DNA (mutant and wild type) may degrade, but as long as at least a single copy of mutant sequence is recovered and amplified in the PCR reactions, it should be possible to detect mutations downstream in the analytical process. Interestingly, sample GP-105, which had an undetectable mutation after incubation with no stabilization buffer, yielded less than 100 copies of total human DNA, suggesting that for a typical 1% mutant population for DNA recovered from stool,^{12,30,31} there was less than a single copy of mutant DNA available for the amplification reaction. Despite the apparent stability of the point mutation markers to DNA degradation, it is nevertheless desirable to mitigate loss of DNA if possible. It has recently been shown¹⁰ that to maximize clinical sensitivity of fecal DNA assays, it is important to maximize the recovery of otherwise scarce amounts of human DNA.

In summary, even under adverse sample handling conditions, 91% of samples (10/11; 95% CI: 59%-100%) that were positive by the point mutation panel remain positive. Under the same conditions, however, the DIA markers suffer an 82% loss in sensitivity (9/11 samples degrade; 95% CI: 48%-98%). All DIA-positive samples (11/11, 100%), and all mutation panel positive samples (11/11, 100%) remained positive after storing for ≥ 36 hours after addition of stabilization buffer to the stool. Transporting samples frozen is a viable means of temporarily neutralizing active nucleases and mitigating degradation of DNA. However, ensuring that samples are frozen immediately and keeping samples frozen during transport to test facilities poses challenges and risks in

a population-based screening application of fecal DNA assays. Our results suggest that addition of stabilization buffer to samples upon collection should provide a robust means of storing (and transporting) samples at room temperature.

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ERRATUM

In the paper entitled “A Novel Method for Rapid Genotypic Identification of Alpha1-antitrypsin Variants” by I. Ferrarotti et al was published in DMP in the issue 2004 Sep;13(3):160–3. The authors’ have notified us about a mistake regarding primer orientation, in *Methods* section. The correct primers are as follows:

P3M: 5' CTTCCAAACCTTCACTCACCCCTGGT 3'
 and P3P: 5' GTCCTCATGGAGCATGGACGGCG 3' for detection of the
 S variant; 5M: 5' GAGCCTTGCTCGAGGCCTGGGATC 3'
 and 5P: 5' CAGAGAAAACATGGGAGGGATTACA 3' for detection of the
 Z variant.