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Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT–PCR

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Accurate identification of lymph node involvement is critical for successful treatment of patients with colorectal carcinoma (CRC). Real-time quantitative RT–PCR with a specific probe and RNA copy standard for biomarker mRNA has proven very powerful for detection of disseminated tumour cells. Which properties of biomarker mRNAs are important for identification of disseminated CRC cells? Seven biomarker candidates, CEA, CEACAM1-S/L, CEACAM6, CEACAM7-1/2, MUC2, MMP7 and CK20, were compared in a test-set of lymph nodes from 51 CRC patients (Dukes' A–D) and 10 controls. Normal colon epithelial cells, primary tumours, and different immune cells were also analysed. The biomarkers were ranked according to: (1) detection of haematoxylin/eosin positive nodes, (2) detection of Dukes' A and B patients, who developed metastases during a 54 months follow-up period and (3) identification of patients with Dukes' C and D tumours using the highest value of control nodes as cutoff. The following properties appear to be of importance; (a) no expression in immune cells, (b) relatively high and constant expression in tumour tissue irrespective of Dukes' stage and (c) no or weak downregulation in tumours compared to normal tissue. CEA fulfilled these criteria best, followed by CK20 and MUC2.

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It is now well established that the earlier the diagnosis of cancer, the better the clinical outcome, through earlier administration of effective and possibly curative treatments (Etzioni *et al*, 2003). For colorectal cancer (CRC) the only curative treatment is surgery. However, even after curative surgery the tumour recurs in many instances. In CRC the prognostic predictor is tumour stage based on histopathologic examination of the resected specimen combined with perioperative findings (Dukes and Bussey, 1958; Lindmark *et al*, 1994; Fleming *et al*, 1997). It has been shown that adjuvant chemotherapy reduces the relative mortality rate by one-third in patients operated for colon cancer in Dukes' Stage C (any TN1-2M0, Stage III) (Moertel *et al*, 1995; Fleming *et al*, 1997). Adjuvant chemotherapy is not routinely given to patients of Dukes' Stage A (T1-2N0M0, Stage I) or Stage B (T3-4N0M0, Stage II) although a substantial number of patients with Dukes' Stage B tumour will die from tumour recurrences. It is considered to be of vital importance to improve the selection criteria in identifying patients who may benefit from adjuvant chemotherapy and intense follow-up protocols.

A key to these efforts is finding ways to specifically identify disseminated tumour cells in regional lymph nodes. Different techniques, for example analysis of multiple haematoxylin/eosin (H&E)-stained sections, immunohistochemistry, gel-based qualitative reverse transcriptase–polymerase chain reaction (RT–PCR) and more recently real-time quantitative RT–PCR (qRT–PCR), and biomarkers including so-called surrogates of cancer have been utilised (Tsavellas *et al*, 2001; Lotze *et al*, 2005). It can now be concluded that real-time qRT–PCR assay for biomarker mRNA, particularly when used with a specific probe and RNA copy standard, is a superior method for micrometastases detection because it is objective, highly sensitive and quantitative, and has a very wide measuring range (Godfrey *et al*, 2001; Ho *et al*, 2004; Öberg *et al*, 2004). Moreover, this technology lends itself to automation and rapid test performance suitable for a clinical setting (Raja *et al*, 2005).

In a recent study, we used real-time qRT–PCR for detection of disseminated tumour cells in lymph nodes of CRC patients using mRNA for two commonly used tumour markers, carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) (Öberg *et al*, 2004). The results were promising, particularly for CEA. However, there might be biomarkers even more suitable for this purpose and neither CEA nor CK20 discriminates between dislocated normal intestinal epithelial cells (iECs) and tumour cells with propensity to metastasize. Three kin molecules to CEA are expressed in the

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colonic epithelium, that is CEA cell adhesion molecule-1 (CEACAM1), CEACAM6 and CEACAM7 (Horst and Wagener, 2004; Frängsmyr *et al*, 1995, 1999). CEACAM6 has a wider cellular distribution than CEA. However, it has been claimed that tumour levels are higher for CEACAM6 than CEA and that CEACAM6 therefore would be the better tumour marker (Ilantzis *et al*, 2002; Jantschkeff *et al*, 2003). CEACAM1 comes with a long (CEACAM1-L) or short (CEACAM1-S) cytoplasmic tail. The long cytoplasmic tail of CEACAM1-L was shown to mediate tumour suppressor function in model systems while the short cytoplasmic tail of CEACAM1-S lacks this property (Hammarström, 1999; Horst and Wagener, 2004). Thus, CEACAM1-S might be a useful biomarker and an increased CEACAM1-S/CEACAM1-L ratio an indicator of tumour transformation. As far as one knows, CEACAM7 shows the same restricted expression pattern as CEA. CEACAM7 mRNA comes in two splice forms (CEACAM7-1 and CEACAM7-2) coding for molecules that differ in the number of extracellular immunoglobulin-like domains. Little is known about their relative expression levels and possible changes in CRC. Mucin 2 (MUC2) is the major mucin in colon (Weiss *et al*, 1996). It is a candidate tumour marker since its expression is restricted to epithelial cells. Finally, matrix metalloproteinase 7 (MMP7)/matrilysin is an interesting candidate biomarker since it is expressed in the invasive front of the primary tumour and MMP7 expression is correlated with capacity to form metastases in CRC transfer experiments (Adachi *et al*, 2001; Yamamoto *et al*, 2003).

The aims of this study were: (1) to determine which properties of a tumour mRNA marker decide if it is suitable for detection of tumour cells in tissues with an excess of immune cells, (2) to find tumour markers that would be complementary to CEA mRNA by improving sensitivity for the identification of disseminated tumour cells in lymph nodes of CRC patients and (3) to find a biomarker for tumour cells with metastasizing capacity.

MATERIALS AND METHODS

Patients

Surgery for CRC was carried out in 51 patients (32 men, 19 women; median age 69 years, range 52–90). Thirty-five tumours were located in colon and 16 in rectum. Seven patients with rectal cancer received preoperative irradiation with 25 Gy. Radical excision of the tumours with wide lymph node dissection was carried out in 42 patients. Nine patients had distant metastases. Five patients received adjuvant chemotherapy. According to Dukes' classification there were six tumours in Stage A (T1–2N0M0, Stage I), 26 in Stage B (T3–4N0M0, Stage II), 10 in Stage C (anyT1–2M0, Stage III) and nine in Stage D (anyTanyNM1, Stage IV). At follow-up after median 54 months (range: 35–68) 19 patients had died from CRC and eight patients had died from noncancer disease. None of the living patients had tumour recurrence.

Controls included seven men and three women (median age 30 years, range: 18–61) undergoing colorectal surgery for ulcerative colitis (UC; $n=6$), Crohn's disease (CD; $n=3$) and rectal prolapse ($n=1$). Informed consent was obtained from the patients. The local Research Ethics Committee of the Medical Faculty, Umeå University, Sweden, approved our study.

Lymph nodes

One to four lymph nodes were dissected from surgically removed specimens and bisected with separate knives under sterile conditions to prevent RNA cross-contamination. One half of each node was fixed in 10% buffered formalin for routine H&E staining and the other half was snap frozen in liquid nitrogen and stored at -70°C until RNA extraction. In all, 95 lymph nodes were collected

from the CRC patients (11 nodes from Dukes' Stage A patients, 55 nodes from Dukes' Stage B patients, 16 nodes from Dukes' Stage C patients and 16 nodes from Dukes' Stage D patients). In the control group 18 nodes were from UC patients, 12 nodes from CD patients and four nodes from the patient with rectal prolapse.

CRC tissue

An approximately $0.5 \times 0.5 \times 0.5$ cm piece was collected from the outer rim of 20 tumour specimens immediately after resection (two Dukes' Stage A, 13 Dukes' Stage B, two Dukes' Stage C and three Dukes' Stage D), snap-frozen and kept at -70°C until RNA extraction.

Epithelial cells from colon tissue

Colonic epithelial cells were isolated from apparently normal tissue constituting the resection margin after surgical removal of tumour in CRC patients and from colon of UC patients subjected to surgical treatment as described earlier (Fahlgren *et al*, 2003b). The isolation procedure yields one fraction enriched in crypt epithelial cells (crypt-iECs) and one fraction enriched in luminal epithelial cells (luminal-iECs).

Cell lines and peripheral blood mononuclear cells

Human cell lines used were: LS174T, T84, HT29, and HCT8 (colon carcinomas), Jurkat and Molt-4 (T-cell lymphomas), CNB6 and KR4 (EBV-transformed B cell lines; a mixture of equal amounts of RNA from the two lines was used in the analyses), U266 (plasmacytoma), U937 (monocyte-like cell line), K562 (erythroblastoid cell line), HL60 (promyelocytic cell line). Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy adults by Ficoll-Isopaque gradient centrifugation. PBMC were *in vitro* activated by incubation with anti-CD3 mAb OKT3 (50 ng ml^{-1}) in HEPES-buffered RPMI1640 supplemented with 0.4% human serum albumin. PBMC from seven individuals were incubated with the stimulus in parallel cultures for 4, 7, 20, 48 and 72 h, washed, pooled and RNA extracted.

RNA extraction

Total RNA was extracted using the Acid Guanidine Phenol Chloroform (AGPC) method by adding 0.5 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol per 25 mg tissue and up to 2.5×10^6 cells in the first homogenization step. Extracted RNA was dissolved in RNase-free water containing RNase inhibitor.

Real-time qRT-PCR

Real-time qRT-PCR assays for CEA, CEACAM6, CEACAM1-S, CEACAM1-L, CEACAM7-1, CEACAM7-2, MUC2, MMP7 and CK20 mRNAs were constructed in the laboratory using the TaqMan EZ technology (Applied Biosystems, Foster City, CA, USA). Specific primer pairs were placed in different exons and a dye-labelled probe was placed over the boundary between the two exons in the amplicon. Assays for all markers except MMP7 have been described (Fahlgren *et al*, 2003a; Forsberg *et al*, 2004; Öberg *et al*, 2004). The sequences for the MMP7 primers and probe were: forward primer 5'-GGGAGGCATGAGTGAGCTAC-3', reverse primer 5'-TCTCCTTGAGTTGGCTTCTAAA-3' and probe 5'-TCTTGAGATAGTCCTGAGCCTGTTCCCA-3'. The reporter dye at the 5'-end of each probe was FAM. The quencher dye at the 3'-end was TAMRA for CEA, CEACAM1-L, CEACAM6, CEACAM7-2, CK20 and MMP7, and MGB for CEACAM1-S, CEACAM7-1 and MUC2. Emission from released reporter dye was monitored by the

ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Wellesley, MA, USA). The RT-PCR profile for all assays except CEACAM7-1 was: 49°C for 2 min, 59°C for 30 min, 94°C for 5 min followed by 45 cycles of 93°C for 20 s and 61°C for 1 min. The RT-PCR profile for CEACAM7-1 was: 49°C for 2 min, 59°C for 30 min, 94°C for 5 min followed by 45 cycles of 93°C for 20 s and 59°C for 1 min. Specific RNA copy standards were prepared as described previously (Fahlgren *et al*, 2003b). Determinations were carried out in triplicates and expressed as copies of mRNA per μ l as determined from parallel RT-PCR of serial dilutions of the RNA copy standard. The concentration of 18S rRNA was determined in each sample by real-time qRT-PCR according to the manufacturer's protocol (Applied Biosystems). As no copy standard is available for the 18S rRNA assay the 18S rRNA content was expressed as arbitrary units defined as the amount of 18S rRNA in 1 pg total RNA extracted from PBMC. Results are expressed as mRNA copies per unit of 18S rRNA.

Statistics

The mRNA expression levels in iECs of normal and inflamed colon were compared using two-tailed Mann-Whitney's rank sum test. Comparisons between mRNA expression levels in tumour vs normal iECs and between levels of different mRNA species in tumour tissue were performed using Kruskal-Wallis' nonparametric one-way ANOVA with Dunn's multiple comparison *post hoc* test. Analyses of correlations between levels of different mRNA species were performed using two-tailed Spearman rank correlation test. A *P*-value <0.05 was considered statistically significant.

RESULTS

Biomarker mRNA levels in colonic epithelial cells and CRC tumours

Levels of CEA, CEACAM1-S/L, CEACAM6, CEACAM7-1/2, MUC2, MMP7 and CK20 mRNAs were assessed in RNA extracted from primary tumours representing all four Dukes' stages and from luminal-iECs and crypt-iECs of apparently normal and inflammatory colon using specific real-time qRT-PCR assays with RNA copy standards. The 18S rRNA concentration was determined in each sample and levels are expressed as mRNA copies/18S rRNA unit allowing direct comparison between the different biomarkers. Statistically significant difference in mRNA levels between iECs from normal and inflammatory colon was only seen for MMP7 (see

legend to Table 1), therefore the two groups were combined in the comparisons against tumour tissue. Table 1 summarises the results. The mRNA levels of the different biomarkers varied considerably within the tumours, from median 107 mRNA copies/18S rRNA unit for CEA to 0.05 for CEACAM7-1. CEA and CEACAM6 mRNAs were expressed at approximately equal levels in tumours and iECs while the mRNA levels for CEACAM7-1/2, CEACAM1-L, MUC2 and CK20 were significantly decreased in the tumour. Only MMP7 mRNA levels were increased in the tumour compared to iECs (Table 1). Table 2 shows the mRNA levels of the biomarkers in four CRC cell lines. Among the biomarkers CEA exhibited the highest expression level and smallest variation between CRC cell lines.

Biomarker mRNA levels in immune cells

Table 2 summarises the results of analysis of polyclonally activated and resting PBMC and seven immune cell lines. Four biomarker mRNAs were not detected or were detected in trace amounts only in one type of immune cells. These were MUC2, CEA, CEACAM7-2 and CK20. Other biomarkers were expressed at high levels in one or several types of immune cells (CEACAM1-L, CEACAM6, MMP7) or at low levels (CEACAM1-S, CEACAM7-1).

Specificity indexes

To rank the different biomarkers with respect to specificity for CRC we calculated two types of specificity indexes: (I) median value in CRC tumours/highest value in any type of immune cell and (II) median value in CRC tumours/highest value of control lymph nodes (Table 3). As can be seen, the two indexes gave similar values with the exceptions of those for CEACAM6 and CEACAM7-2. CEA was the most specific biomarker followed by CK20. CEACAM6 showed better specificity with index II than index I, while the reverse was true for CEACAM7-2. Indexes for three biomarkers (CEACAM1-L, CEACAM7-1, MMP7) showed no specificity for CRC.

Biomarker mRNA levels in lymph nodes of CRC patients and controls

A total of 129 lymph nodes of 51 CRC patients and 10 controls were analysed for mRNA levels of the biomarkers. Figures 1–3 summarise the results. For each individual only the lymph node

Table 1 Expression levels of mRNAs for CEA, CEACAM1, CEACAM6, CEACAM7, MUC2, MMP7, and CK20 in primary colorectal tumours compared to control colonic epithelial cells

mRNA species	Tumour		Crypt iEC ^a			Luminal iEC		
	Median ^b	IQR ^c	Median	IQR	<i>P</i> -value ^d	Median	IQR	<i>P</i> -value
CEA	107	39–1207	261	225–537	NS	393	212–769	NS
CEACAM6	20	6.5–55	28	14–35	NS	38	13–116	NS
CEACAM1-S	5.1	2.5–14	19	12–34	<0.01	28	12–72	<0.01
CEACAM1-L	1.4	0.43–6.4	16	8.0–40	<0.001	22	10–40	<0.001
CEACAM7-1	0.05	0.005–0.37	0.90	0.67–2.9	<0.001	1.9	1.2–6.0	<0.001
CEACAM7-2	9.0	0.94–32	302	162–468	<0.001	483	361–582	<0.001
MUC2	0.66	0.04–3.4	32	25–56	<0.001	33	17–77	<0.001
MMP7	1.7	0.85–4.3	0.09	0.01–0.25	<0.01	0.09	0.003–16	<0.05
CK20	14	5.5–40	295	178–675	<0.001	162	104–377	<0.001

^aiEC = intestinal epithelial cells. ^bMedian mRNA copies/18S rRNA unit of 20 CRC tumour samples (2 Dukes' Stage A, 13 Dukes' Stage B, 2 Dukes' Stage C and 3 Dukes' Stage D), 14–20 crypt iEC samples and 10–20 luminal iEC samples. Equal numbers of iEC samples were derived from the apparently healthy resection margin of colon from patients operated for CRC and from colon of UC patients. The mRNA expression levels for any of the markers in iEC from normal colon of CRC patients and UC colon, respectively, did not differ significantly from each other with the exception of MMP7 mRNA. MMP7 mRNA levels were significantly higher (*P*<0.01) in the iECs from UC patients compared to iEC from the resection margin of CRC patients. MMP7 values for iEC in the Table include data from 10 CRC and 10 UC patients. ^cIQR = interquartile range from the 25 to the 75 percentile. ^d*P*-value obtained by comparing mRNA expression levels in tumours with those in crypt iECs, and luminal iECs using Kruskal-Wallis nonparametric one-way ANOVA with Dunn's multiple comparison *post hoc* test. NS = not significant, that is *P*-value >0.05.

Table 2 Expression levels of mRNA for CEA, CEACAM1, CEACAM6, CEACAM7, MUC2, MMP7, and CK20 in CRC cell lines and different types of immune cells

Cells	Origin	Biomarker mRNA								
		CEA	CEACAM6	CEACAM1-S	CEACAM1-L	CEACAM7-1	CEACAM7-2	MUC2	MMP7	CK20
HT29	Colon iEC	32 ^a	43	22	17	0.003	0.3	0.01	53	85
LS174T	Colon iEC	328	81	1.2	1.4	0.5	38	4.3	3.4	0.02
T84	Colon iEC	33	1.0	0.9	0 ^b	0.003	0.07	0.5	2.3	33
HCT8	Colon iEC	32	0.5	0.003	0	0	0.1	0.02	0.2	0.05
PBMC		0	0.05	0	0.3	0.01	0	0	0.001	0
Act. PBMC		0	0	0.001	0.8	0	0	0	2.0	0
Jurkat	T cell	0	0	0.004	0.1	0	0	0	0.004	0
Molt-4	T cell	0	0	0.002	0.05	0	0	0	0	0
CNB6+KR4	B cell	0	0	0.008	1.1	0.05	0	0	0.6	0
U266	Plasma cell	0	0	0.001	0.02	0	0	0	0	0
U937	Monocyte	0.005	0.3	0.002	0.03	0	0	0	0.9	0.003
HL60	Granulocyte	0	1.8	0	ND	0	0.001	ND	0	0
K562	Pre-erythrocyte	0	0	0.006	0.08	0	0	0	0	ND

^aExpression level of the indicated mRNA species expressed as mRNA copies/18S rRNA unit. ^bValues below 0.001 mRNA copies/18S rRNA unit are recorded as 0. ND = not determined.

Table 3 Specificity indexes for tumour marker mRNAs

mRNA species	Median CRC tumour value/highest value of any immune cell type	Median CRC tumour value/highest value of control lymph nodes
CEA	21 400	53 500
CEACAM6	11	2000
CEACAM1-S	638	39
CEACAM1-L	1.3	0.4
CEACAM7-1	1.0	6.3
CEACAM7-2	9000	90
MUC2	> 660	942
MMP7	0.9	1.1
CK20	4667	15 555

with the highest level of the indicated mRNA species is shown. For each biomarker, a cutoff level is set at the highest control node.

CEA mRNA showed excellent discrimination between nodes from CRC patients and controls (Figure 1). Practically all CRC nodes were above the cutoff level independent of Dukes' Stage and all H&E positive lymph nodes had very high CEA mRNA levels. Moreover, the three Dukes' B patients that have died from their CRC within the 54 months follow-up period displayed highly elevated CEA mRNA levels (arrows in figure). The single Dukes' A patient that has died from her CRC showed a borderline value (arrow).

CEACAM1-S mRNA (Figure 2A) showed some discrimination between CRC and controls. Nodes of 1/6 Dukes' A, 5/24 Dukes' B, 7/10 Dukes' C and 2/8 Dukes' D patients were above cutoff and all but one H&E positive nodes were identified as positive by CEACAM1-S mRNA level. Moreover, two of the three Dukes' B patients, but not the Dukes' A patient, that have died from their CRC within the follow-up period were identified. Note also that the total range of values is much smaller for CEACAM1-S than for CEA, that is four and seven orders of magnitude, respectively, and that the CEACAM1-S levels in controls are almost 100 times higher.

No discrimination between CRC and control nodes was seen with CEACAM1-L mRNA (Figure 2B). The CEACAM1-L mRNA levels of control nodes were very high, which is consistent with the high levels of CEACAM1-L mRNA in immune cells (Table 2).

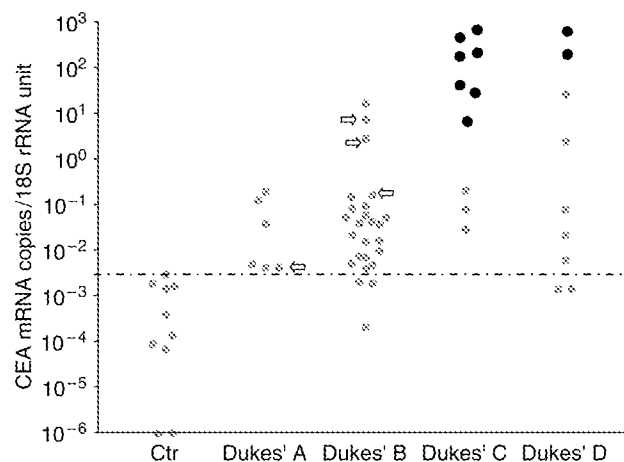


Figure 1 CEA mRNA levels in lymph nodes as determined by real-time qRT-PCR for CEA mRNA and 18S rRNA content in the samples. Each patient is represented by the lymph node with the highest CEA mRNA level (dots). Arrows indicate the four patients with Dukes' Stage A and B tumours, who had died from CRC during the 54 months (range: 35–68 months) follow-up time. Large black dots indicate lymph nodes that had tumour cells identified by H&E staining and small grey dots indicate H&E negative nodes. Results updated from Öberg *et al*, 2004.

CEACAM7-1 mRNA was not a useful marker (Figure 2C) while CEACAM7-2 mRNA, which was expressed at about 100 times higher concentration, displayed some discriminating power between CRC patients and controls (Figure 2D). However, several H&E positive nodes were missed and one of the three Dukes' B patients and the Dukes' A patient that have died from CRC within the follow-up period were missed.

MUC2 mRNA showed good discrimination between CRC nodes and control nodes comparable to that of CEA mRNA. All but one of the H&E positive nodes and all three Dukes' B patients who had died from CRC had values above the cutoff (Figure 3A). Interestingly, MUC2 mRNA values are very low in controls and the range of values in CRC is quite large.

Similarly, CK20 mRNA showed good discrimination between nodes from CRC patients and controls (Figure 3B). All H&E positive nodes as well as the three Dukes' B patients who have died

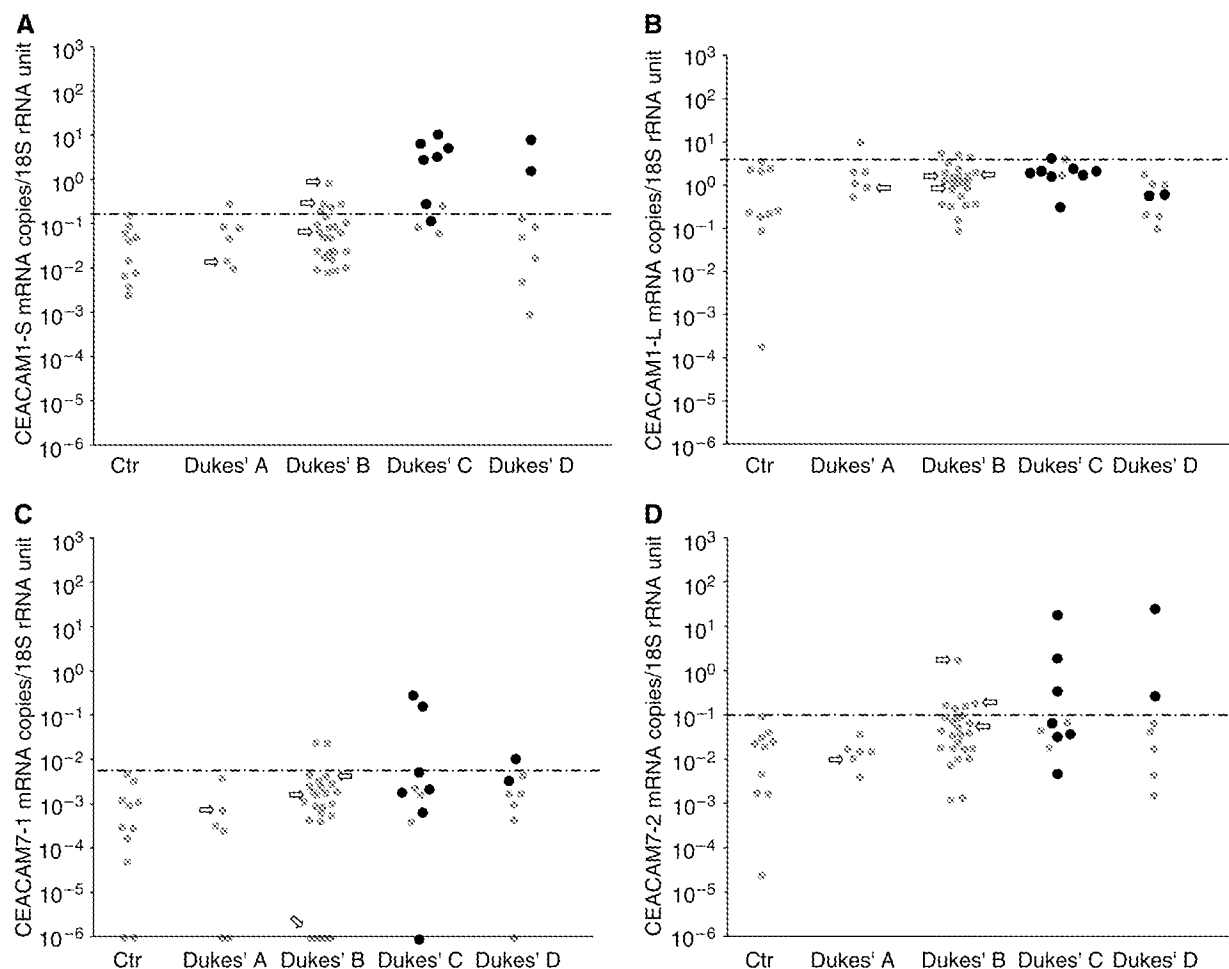


Figure 2 CEACAM1-S (A), CEACAM1-L (B), CEACAM7-1 (C), and CEACAM7-2 (D) mRNA levels in lymph nodes of CRC and control patients. Each patient is represented by the lymph node with the highest level of the respective mRNA species. For explanation of dots and arrows see legend to Figure 1.

from CRC during the follow-up period were identified as positive. CK20 mRNA levels in CRC nodes varied over a wide range and control nodes were low.

CEACAM6 mRNA displayed fairly good ability to discriminate between CRC patients and controls (Figure 3C). However, only two of the three Dukes' B patients that have died from their CRC within the follow-up period had elevated CEACAM6 levels. All H&E positive nodes displayed CEACAM6 mRNA levels above cutoff.

MMP7 mRNA displayed poor discriminating power between CRC patients and controls (Figure 3D).

Correlation between different biomarker mRNA levels in lymph nodes of CRC patients and controls

The four biomarker mRNAs which showed the best discriminating power between CRC and control nodes and which were not appreciably expressed in immune cells namely CEA, CEACAM7-2, CK20 and MUC2 were analysed by pair-wise comparisons of biomarker levels in all 129 lymph nodes. Each Dukes' Stage and controls were also analysed separately. Table 4 shows correlation coefficients (r -values) and significance levels (P -values) for all six comparisons. Biomarker mRNA values were significantly correlated if all nodes were compared with r -values ranging from 0.47 to 0.76. When the different stages of CRC were analysed separately it was noted that lymph nodes from Dukes' C and Dukes' D patients showed r -values ≥ 0.5 and P -values ≤ 0.05 for all marker combinations except one indicating that it was the same cells that expressed

the four markers. The comparisons furthermore demonstrate that CEACAM7-2 is also expressed in a separate cell population normally present in lymph nodes. The results also indicate that control nodes harbour low numbers of two cell populations of epithelial origin, one expressing CEA, CK20 and CEACAM7-2 and another expressing MUC2, CK20 and CEACAM7-2.

DISCUSSION

In this study, we have explored the possibility of using real-time qRT-PCR as an alternative, or adjunct, to the classical H&E staining method for detection of disseminated tumour cells in regional lymph nodes. There are several reasons to search for alternatives. Firstly an inherent problem with histochemical as well as immunohistochemical methods is that of sampling. Usually, only one or a few 6 μ m thick sections are analysed covering <1% of the lymph node volume. To analyse the number of sections that would be needed to properly cover the node becomes impossible in practice. In the qRT-PCR study reported here, we extracted RNA from one half of the lymph node. A second problem specific for H&E is to decide on the basis of morphology alone whether a small number of cells indeed are tumour cells. Thirdly, and in contrast to H&E and immunohistochemistry, the qRT-PCR assay is very sensitive and objective and has a very large measuring range. With the appropriate choice of primers and probes, it can be made highly specific for the chosen marker mRNA. Moreover, since

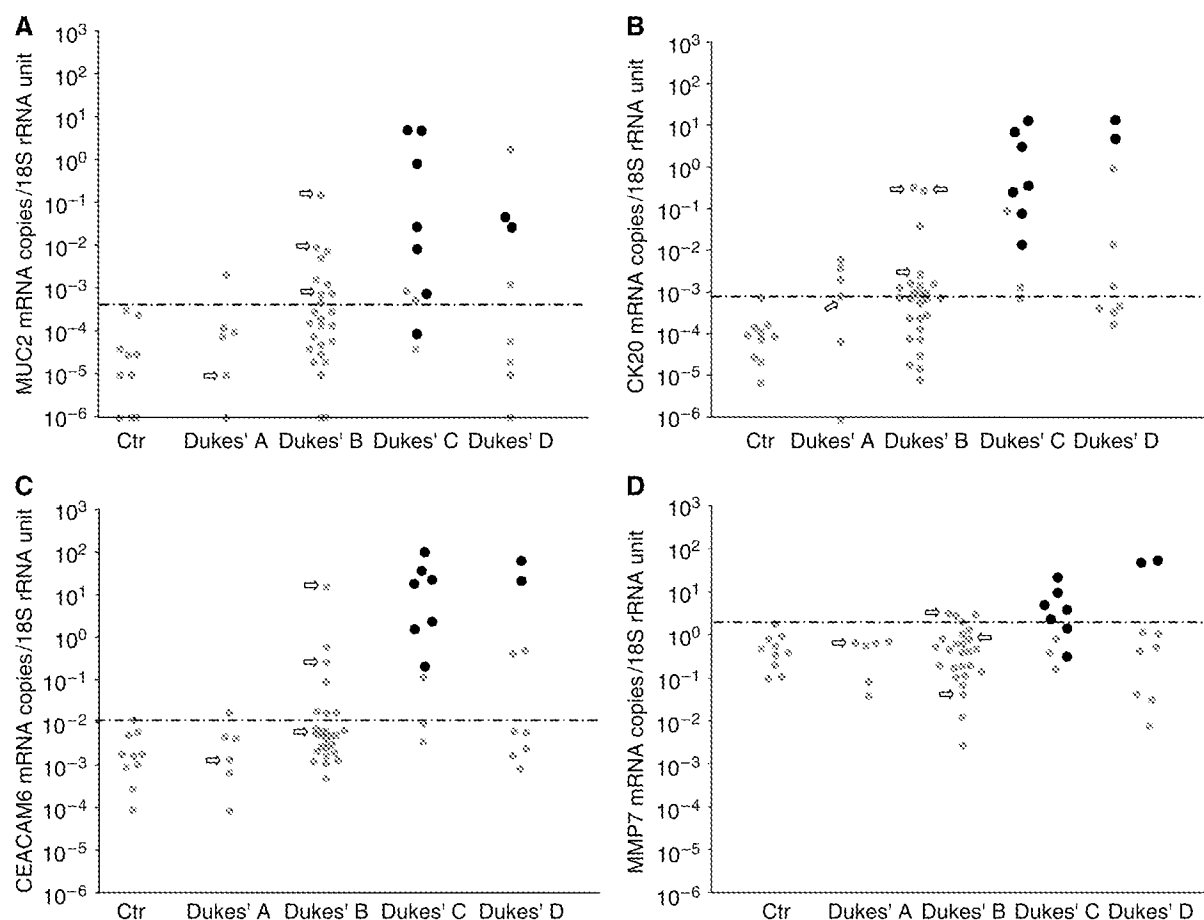


Figure 3 MUC2 (A), CK20 (B), CEACAM6 (C), and MMP7 (D) mRNA levels in lymph nodes of CRC and control patients. Each patient is represented by the lymph node with the highest level of the respective mRNA species. For explanation of dots and arrows see legend to Figure 1.

Table 4 Correlation between expression levels of biomarker CEA, CEACAM7-2, CK20 and MUC2 mRNAs in lymph nodes of CRC patients and controls

Compared mRNA species	All lymph nodes		Controls		Dukes' A		Dukes' B		Dukes' C		Dukes' D	
	<i>r</i> ^a	<i>P</i> -value ^a	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
CEA vs CK20	0.76	<0.0001	0.47	0.005	0.80	0.005	0.58	<0.0001	0.70	0.004	0.76	0.0006
MUC2 vs CK20	0.62	<0.0001	0.50	0.004	0.62	0.04	0.45	0.001	0.69	0.007	0.60	0.05
CEA vs MUC2	0.71	<0.0001	0.33	NS	0.60	NS	0.55	<0.0001	0.78	0.0006	0.80	0.003
CEA vs CEACAM7-2	0.62	<0.0001	0.64	<0.0001	0.42	NS	0.40	0.004	0.54	0.03	0.89	0.0003
CEACAM7-2 vs CK20	0.47	<0.0001	0.53	0.001	0.37	NS	0.16	NS	0.52	0.05	0.85	0.002
CEACAM7-2 vs MUC2	0.55	<0.0001	0.43	0.02	0.44	NS	0.43	0.003	0.41	NS	0.80	0.005

^a*r*- and *P*-values obtained by pair-wise comparing the expression levels of the indicated mRNA species in lymph nodes of CRC patients and controls using two-tailed Spearman rank correlation test. Numbers of lymph nodes in the analysis were 120–129 for all lymph nodes, 33–34 for controls, 10–11 for Dukes' A patients, 48–52 for Dukes' B patients, 15–16 for Dukes' C patients and 12–16 for Dukes' D patients. NS = not significant, *P*-value > 0.05.

analyses are performed at the mRNA level instead of at the protein level positive results are likely to reflect the presence of living tumour cells. It has been shown that dendritic cells/macrophages can transport protein components from apoptotic epithelial cells to lymph nodes (Bonnotte *et al*, 2000; Huang *et al*, 2000). A yet unresolved question in using qRT-PCR for the purpose of detecting disseminated tumour cells in regional lymph nodes is, however, which biomarker mRNA to use.

A major goal of this study was to determine which properties of a tumour marker mRNA are of importance for the detection of disseminated colon tumour cells in the presence of an excess of immune cells for example in regional lymph nodes and blood. We,

therefore, investigated a number of biomarkers, that are expressed in colon adenocarcinoma cells and compared their expression levels in a test-set of lymph nodes from CRC patients of different Dukes' stages and controls as well as in primary tumours, in epithelial cells of normal and inflamed colon and in different types of immune cells.

We found that CEACAM1-S/L, CEACAM7-1/2, MUC2 and CK20 mRNAs were expressed at lower levels in the primary tumour compared to normal colon. CEACAM1 and CEACAM7 mRNAs have previously been shown to be downregulated in CRC using semiquantitative methods (Neumaier *et al*, 1993; Nollau *et al*, 1997). In contrast, neither CEA nor CEACAM6 mRNA levels were

decreased in tumour tissue. MMP7 was the only marker that exhibited increased levels in tumours. However, the levels were still low compared to CEA. CEA and CEACAM1-S levels displayed the smallest variation between individual primary tumour tissue samples and there was no tendency for changes in relation to Dukes' stages for these two markers. Of all biomarkers CEA mRNA was expressed at the highest levels in tumours followed by CEACAM6. The high levels of these two markers could possibly be explained by the fact that CEA and CEACAM6 are expressed both in columnar epithelial cells and goblet cells in normal colon while CEACAM1 and CEACAM7 are expressed in columnar epithelial cells only and MUC2 in goblet cells only (Weiss *et al*, 1996; Frängsmyr *et al*, 1999).

It can be argued that no or only marginal expression of the biomarker in immune cells should be of utmost importance for successful detection of tumour cells in lymph nodes. Indeed, three of the four biomarkers that were expressed in only trace amounts in immune cells, that is CEA, CK20 and MUC2, showed good discriminatory capacity between nodes from CRC patients and controls. Owing to its low expression in immune cells one would have predicted excellent discriminatory capacity also by CEACAM7-2 but this was not the case. Possibly CEACAM7-2 is expressed in some additional cell type in lymph nodes, for example endothelial cells or stromal cells.

CEA mRNA showed excellent separation between CRC patients and controls. MUC2 and CK20 mRNAs had almost the same discriminating power as CEA mRNA. The Dukes' B patients who developed metastatic disease during the follow-up period were all detected by the three markers but only for CEA were all Dukes' C nodes above the cutoff value. Moreover, one H&E positive lymph node was missed by the MUC2 mRNA assay.

It was somewhat unexpected that MUC2 gave essentially the same result as CEA although in normal colon MUC2 is confined to goblet cells and not expressed in columnar epithelial cells. Does all CRC tumour disseminated to the lymph node contain goblet cell-like elements? We do not know whether this is the case. Possibly MUC2 expression can be induced by external agents in CRC tumour cells. It was recently shown that bile acids induce MUC2

overexpression in human colon carcinoma cells (Song *et al*, 2005). Furthermore, we found that the inflammatory state of the small intestinal mucosa in patients with active celiac disease is associated with ectopic production of MUC2 by enterocytes (Forsberg *et al*, 2004). Thus, the cytokine milieu in the nodes might upregulate MUC2 expression in the iECs.

Although CEA and CEACAM6 behaved very similar CEA had a higher discriminatory power. This was due to CEA that, contrary to expectation, had higher expression level in tumour cells than CEACAM6 and that CEACAM6 was expressed in myeloid immune cells with the risk that tumour cells expressing fairly low levels of this marker would drown in the immune cell background of the node.

The two markers for tumour cell 'aggressiveness' studied here, that is MMP7 and CEACAM1-S, were not suitable for analysis of lymph nodes. MMP7 because its expression was induced in activated T lymphocytes, a cell type which is likely to be prominent in lymph nodes. CEACAM1-S was not suitable because (1) it was expressed in several types of immune cells although at low levels and (2) the very high expression level of CEACAM1-L in immune cells precludes meaningful calculation of CEACAM1-S: CEACAM1-L ratios in lymph nodes.

In conclusion, the finding that three independent biomarker mRNAs, CEA, MUC2 and CK20, gave almost the same results with highly selective expression in cells of epithelial origin strongly support the notion that disseminated tumour cells can be successfully detected in regional lymph nodes by this technique. CEA mRNA appears to be the best choice as single marker due to its remarkably high expression level in colorectal tumour cells.

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Stool DNA and Occult Blood Testing for Screen Detection of Colorectal Neoplasia

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Abstract

Background—Stool DNA testing is a new approach to colorectal cancer detection. Few data are available from the screening setting.

Objective—To compare stool DNA and fecal blood testing for detection of screen-relevant neoplasia (curable-stage cancer, high-grade dysplasia, or adenomas >1 cm).

Design—Blinded, multicenter, cross-sectional study.

Setting—Communities surrounding 22 participating academic and regional health care systems in the United States.

Participants—4482 average-risk adults.

Measurements—Fecal blood and DNA markers. Participants collected 3 stools, smeared fecal blood test cards and used same-day shipment to a central facility. Fecal blood cards (Hemoccult and HemoccultSensa, Beckman Coulter, Fullerton, California) were tested on 3 stools and DNA assays on 1 stool per patient. Stool DNA test 1 (SDT-1) was a precommercial 23-marker assay, and a novel test (SDT-2) targeted 3 broadly informative markers. The criterion standard was colonoscopy.

Results—Sensitivity for screen-relevant neoplasms was 20% by SDT-1, 11% by Hemoccult ($P = 0.020$), 21% by HemoccultSensa ($P = 0.80$); sensitivity for cancer plus high-grade dysplasia did not differ among tests. Specificity was 96% by SDT-1, compared with 98% by Hemoccult ($P < 0.001$) and 97% by HemoccultSensa ($P = 0.20$). Stool DNA test 2 detected 46% of screen-relevant neoplasms, compared with 16% by Hemoccult ($P < 0.001$) and 24% by HemoccultSensa ($P < 0.001$). Stool DNA test 2 detected 46% of adenomas 1 cm or larger, compared with 10% by Hemoccult ($P < 0.001$) and 17% by HemoccultSensa ($P < 0.001$). Among colonoscopically normal patients, the positivity rate was 16% with SDT-2, compared with 4% with Hemoccult ($P = 0.010$) and 5% with HemoccultSensa ($P = 0.030$).

Limitations—Stool DNA test 2 was not performed on all subsets of patients without screen-relevant neoplasms. Stools were collected without preservative, which reduced detection of some DNA markers.

Conclusion—Stool DNA test 1 provides no improvement over HemoccultSensa for detection of screen-relevant neoplasms. Stool DNA test 2 detects significantly more neoplasms than does Hemoccult or HemoccultSensa, but with more positive results in colonoscopically normal patients. Higher sensitivity of SDT-2 was particularly apparent for adenomas.

Colorectal cancer remains the second most common cause of death among the types of cancer (1). Although screening reduces colorectal cancer mortality (2–6), observed reductions have been modest (6, 7) and more than one half of adults in the United States have not received screening (8). More accurate, user-friendly, and widely distributable tools have the potential to improve screening effectiveness, acceptability, and access.

Several molecular approaches to screening stool for colorectal cancer have been studied and reviewed (9, 10), and stool DNA testing has been jointly endorsed by the American Cancer Society, the U.S. Multi-Society Task Force on Colorectal Cancer, and the American College

of Radiology (11). The advantages of stool DNA testing include noninvasiveness, absence of bowel preparation or dietary restrictions, and ease of access via mail courier. However, the reported accuracy of stool DNA tests for the detection of colorectal neoplasia varies. In clinical studies that used different assays and selected groups (12–20), sensitivities ranged from 62% to 100% for colorectal cancer and 27% to 82% for advanced adenomas, with specificities ranging from 82% to 100%. In the only reported multi-center study on asymptomatic average-risk patients (21), a precommercial multitarget DNA assay (SDT-1, a prototype of PreGenPlus, EXACT Sciences, Marlborough, Massachusetts) detected 52% of cases of colorectal cancer, compared with 13% by Hemoccult ($P = 0.003$), at specificities of 94.4% and 95.2%, respectively.

The accuracy of stool DNA testing is influenced by both biological and technical factors. A panel of markers must be used to accommodate the molecular heterogeneity of colorectal neoplasia, and marker selection critically affects discrimination (9). Unlike occult bleeding, which is intermittent (22), DNA markers seem to be shed continuously by exfoliation (23). Thus, the multiple stool sampling practiced with fecal occult blood tests may not be necessary with stool DNA tests. However, recovery of the minute quantities of human DNA and assay of tumor-specific DNA alterations from stool present technical challenges and require exquisite laboratory sensitivity to achieve optimal detection rates.

Our primary aim was to compare the precommercial stool DNA test (SDT-1), which was studied by Imperiale and colleagues (21), with widely used fecal occult blood tests for the detection of screen-relevant neoplasia, defined as curable-stage colorectal cancer (no distant metastases), high-grade dysplasia, or adenomas larger than 1 cm. A secondary aim was to explore neoplasm detection by another stool DNA test 2 (SDT-2), which uses a more broadly informative marker panel.

Methods

Table 1 lists the genes used in our test panels and defines several key terms.

Design

We conducted this multicenter, prospective, triple-blinded trial, targeting average-risk persons, from 2001 to 2007. A group of national experts on colorectal cancer screening advised on study design, and institutional review boards at each site approved the study. Because we did not know the effect of diet and medications on DNA assays, patients were randomly assigned at entry to group A (restriction of red meat and therapeutic doses of nonsteroidal anti-inflammatory drugs for 3 days before and during stool collections) or group B (no such restrictions). All patients were asked not to ingest vitamin C for the 3 days before and during stool collections. For the companion test, we chose Hemoccult (Beckman Coulter, Fullerton, California), the most widely used fecal occult blood test, which was used in the trials that established the benefit of screening for fecal occult blood (2–4). As a second companion test, we chose the next-generation guaiac test HemoccultSensa (Beckman Coulter). We compared fecal blood results from 3 stools per patient with stool DNA on 1 stool. Experienced technicians performed stool DNA and occult blood testing in separate central laboratories without knowledge of clinical findings or the results of other tests. All

patients who completed stool collections also had colonoscopy, which served as the criterion standard. We did not have access to data until after they had been analyzed by statisticians and released by a data monitoring board.

Participants

We recruited asymptomatic persons age 50 to 80 years who were at average risk for colorectal cancer from communities surrounding 22 participating academic and regional health care systems through direct mail and multi-media advertisements. The exclusion criteria were structural colorectal evaluation (endoscopic or radiographic) within 10 years; fecal blood testing within 1 year; overt rectal bleeding within 1 month; previous colorectal resection; aerodigestive cancer within 5 years; inability to stop therapeutic doses of nonsteroidal anti-inflammatory drugs or anticoagulants; coagulopathy; contraindications to colonoscopy; chemotherapy within 3 months; high-risk conditions for colorectal cancer, such as familial adenomatous polyposis, the Lynch syndrome, or other cancer syndromes; previous colorectal cancer or adenoma; inflammatory bowel disease; or more than 2 first-degree relatives with colorectal neoplasia. Study assistants at each site registered participants and randomly assigned them by using a Web-based management system; distributed fecal blood test cards, stool collection containers, and colonoscopy preparation materials; and provided instructions.

Stool Collection and Processing

Patients collected 3 stools by using plastic buckets mounted to the toilet seat. Promptly after each individual collection, patients smeared stool onto both windows of their Hemoccult and HemoccultSensa cards and then express-shipped smeared cards and the whole stool (sealed in a bucket in an insulated container cooled with ice packs) to the Mayo Clinic in Rochester, Minnesota. We froze the first stool from each participant whole at -80°C on receipt and sent it in batches on dry ice to EXACT Sciences (Marlborough, Massachusetts) for DNA assay; each of the subsequent 2 stools were archived in aliquots at -80°C . If the first stool weighed less than 30 g or was received more than 48 hours after defecation, it was rejected for DNA analysis and the second or third stool (if it met inclusion criteria) was sent for DNA assay.

Stool Assays

DNA Testing—All assays were polymerase chain reaction–based and were run at EXACT Sciences. Stool DNA test 1 was performed as described in Imperiale and colleagues' study (21). The marker panel for SDT-1 included 21 tumor-specific point mutations (3 on the *K-ras* gene, 10 on the *APC* gene, and 8 on the *p53* gene); the microsatellite-instability marker BAT-26; and long DNA, a marker for delayed apoptosis, which is characteristic of exfoliated neoplastic colonocytes (12). For SDT-2, sequence-specific DNA markers were detected by acrylamide gel electrophoresis, as described by Whitney and colleagues (24); the panel consisted of 3 tumor-specific markers broadly informative for both colorectal cancer and adenomas (25): *K-ras* mutations, scanning of *APC* mutator cluster regions, and methylation of the vimentin gene. We used methods described elsewhere to detect mutant *K-ras* (12), *APC* scanning (25), and vimentin gene methylation (20) assays. We defined any

positive component marker result according to the manufacturer's preestablished criteria as a positive test result.

Occult Blood Testing—The manufacturer that developed the Hemoccult and HemoccultSensa cards, without rehydration, trained technicians on-site at the Mayo Clinic. As recommended by the manufacturer, the technicians added the catalyst solution to cards stored at ambient temperature within 48 to 72 hours of collection. We defined a spreading (enlarging) blue color in 60 seconds in any window of the cards as a positive result and any other result as negative.

Colonoscopy

After cathartic preparation, experienced endoscopists performed colonoscopy in all patients. If the examination did not reach the cecum or inspected less than 90% of the mucosa, the patient was disqualified. Endophotographs documented cecal intubation, and the size and location of all lesions were recorded. Costs not covered by third parties were reimbursed by study funding.

Pathologic Examination

Local pathologists examined all endoscopically or surgically sampled lesions. A gastrointestinal pathologist at the coordinating site reexamined all lesions to confirm diagnosis. Classification discrepancies of screen-relevant neoplasms were adjudicated by a second expert pathologist. We categorized patients with multiple neoplasms according to the most advanced lesion. For assay of markers in screen-relevant neoplasms, DNA was extracted from microdissected tissue.

Statistical Analysis

We calculated sample size to ensure adequate power to detect differences in sensitivity comparisons. We powered the study to ensure an adequate number of cases of curable-stage colorectal cancer and high-grade dysplasia and assumed their combined prevalence to be at least 1.5%. A sample size of 2900 would yield an expected 43 curable-stage cancer or high-grade dysplasia cases, which would provide 90% power to detect a 35% improvement in sensitivity of SDT-1 over the Hemoccult test by using a 2-sided McNemar test with $\alpha = 0.05$ (assuming Hemoccult sensitivity of 25%). The protocol specified interim analyses at one half and three quarters of full enrollment to see whether it was necessary to stop the study early if test sensitivities differed significantly or to revise sample size requirements on the basis of observed prevalence of the target lesion. At the first interim analysis, lesion prevalence was lower than expected, and we readjusted the sample size to 4434 patients. However, before we completed enrollment, the manufacturer altered the SDT-1 assay, which prompted an unplanned interim analysis after 2497 patients. On the basis of these interim results, we stopped SDT-1 testing and began doing the SDT-2 test.

To accomplish a secondary aim of this trial (to see whether restricting diet and medication affects the specificity of the SDT test), we randomly assigned persons to pretest restrictions or no restrictions. The sample size calculated for the sensitivity comparison provided 85%

power to detect a 4% difference in specificity between randomization groups. Because SDT specificity was the same in both groups, we pooled the results for all analyses.

We included all patients tested with SDT-1. We compared stool test sensitivities and specificities by using the McNemar test. We used a chi-square test or the Fisher exact test to compare baseline characteristics between cohorts and assay performance in subsets of patients. All *P* values are 2-sided.

Per agreement with EXACT Sciences, we did the SDT-2 test on all patients with cancer, high-grade dysplasia, and adenomas larger than 2 cm from the full enrollment period as well as on a random sample of 50 patients with 1- to 2-cm adenomas and 75 with normal colonoscopy results. To estimate the population-level sensitivity for the SDT-2 test, we used all case patients tested with SDT-2 and reweighted the calculation to be proportional to the observed prevalence of each screen-relevant neoplasia category in the entire population with screen-relevant neoplasias. Because we did not do the SDT-2 test on all subsets without screen-relevant neoplasia, we could not calculate specificity for screen-relevant neoplasia. To compare test positivity rates in patient subsets, we used the McNemar test.

Role of the Funding Source

The National Cancer Institute funded this study and monitored conduct. EXACT Sciences performed DNA assays at no cost, and Beckman Coulter provided Hemocult and HemocultSensa cards at no cost. EXACT Sciences limited SDT-2 coverage to screen-relevant neoplasms and a subset of normal control participants. Neither company influenced study oversight, data analysis, or reporting.

Results

Patients

Of the 4482 persons enrolled, 3764 (84%) were evaluable. We excluded 545 patients because of cancellations, protocol violations, or ineligibility; 171 because of incomplete colonoscopies; and 2 because of distant metastases (Figure 1). Table 2 shows demographic and colorectal lesion characteristics. We found screen-relevant neoplasms in 290 (7.7%) patients; 39 had nonmetastatic cancer or high-grade dysplasia and 251 had adenomas that were 1 cm or larger. Major complications from colonoscopy occurred in 4 patients; no procedure-related deaths were reported.

Occult Blood Testing: Hemocult versus HemocultSensa

Detection sensitivities for the 290 screen-relevant neoplasms found among all 3764 evaluable participants were 10% (95% CI, 7% to 13%) with Hemocult and 18% (CI, 13% to 22%) with HemocultSensa ($P < 0.001$). Based on all 3474 participants without screen-relevant neoplasia, the Hemocult specificity of 98% (CI, 98% to 99%) was slightly higher than that of HemocultSensa (97% [CI, 96% to 97%]) ($P < 0.001$).

Hemocult and HemocultSensa positivity rates for the 39 patients with colorectal cancer or high-grade dysplasia were 33% (CI, 19% to 48%) and 44% (CI, 28% to 59%), respectively

($P = 0.100$). For the 251 patients with adenomas 1 cm or larger, the positivity rates were 6% (CI, 3% to 9%) versus 14% (CI, 9% to 18%) ($P = 0.001$).

Stool DNA versus Occult Blood Testing

SDT-1 versus Occult Blood Testing—Based on the first 2497 evaluable participants (Table 3), the sensitivity of SDT-1 for screen-relevant neoplasia was higher than that of Hemoccult (20% [CI, 14% to 26%] vs. 11% [CI, 6% to 16%]; $P = 0.020$) but not that of HemoccultSensa (21% [CI, 15% to 27%]; $P = 0.80$). For all target lesion groupings, specificities were slightly but significantly lower for SDT-1 than for Hemoccult but not HemoccultSensa, and the positive likelihood ratios for SDT-1 were lower than for either Hemoccult or HemoccultSensa for the more advanced groupings of screen-relevant neoplasms (Table 3).

Based on test positivity data in subsets of screen-relevant neoplasms (Table 4), SDT-1 had higher detection rates than Hemoccult for 1- to 2-cm adenomas but not for any other subset. Stool DNA test 1 had a lower positivity rate for detecting invasive cancer than did HemoccultSensa (25% [CI, 5% to 57%] vs. 75% [51% to 100%]; $P = 0.010$).

SDT-2 versus Occult Blood Testing—Table 3 shows the sensitivity of SDT-2, Hemoccult, and HemoccultSensa for screen-related neoplasia. The weighted estimate of SDT-2 sensitivity for screen-relevant neoplasms was 40% (CI, 32% to 49%), compared with the observed Hemoccult and HemoccultSensa sensitivities of 11% (CI, 6% to 16%) and 21% (CI, 15% to 27%), respectively (Table 3). Table 4 shows the test positivity rates for subgroups of colorectal lesions.

For adenomas 1 cm or larger, the positivity rate was 46% (CI, 35% to 54%) by SDT-2 versus 10% (CI, 4% to 15%) by Hemoccult ($P < 0.001$) and 17% (CI, 9% to 24%) by HemoccultSensa ($P < 0.001$). Neoplasm site did not affect detection by SDT-2; however, detection rates were lower for lesions proximal to the splenic flexure than for distal lesions with both Hemoccult (9% [CI, 1% to 16%] vs. 21% [CI, 12% to 30%]; $P = 0.060$) and HemoccultSensa (13% [CI, 4% to 21%] vs. 31% [CI, 22% to 41%]; $P = 0.010$). Study comparisons are based on a single stool per patient for SDT-2 and 3 stools per patient for fecal blood tests; differences in test performance are larger if fecal blood test results are analyzed on fewer than 3 stools per patient (Figure 2). For patients with normal colonoscopy results, the positivity rate was 16% (CI, 8% to 24%) by SDT-2, compared with 4% (CI, 1% to 11%) by Hemoccult ($P = 0.010$) and 5% (CI, 1% to 13%) by Hemoccult-Sensa ($P = 0.030$).

Frequencies of DNA Markers in Neoplastic Tissue

Nearly all of the tissues analyzed from screen-relevant neoplasms contained at least 1 marker from the SDT-2 panel; fewer than two thirds of the tissues contained SDT-1 markers (Table 5).

Negative Stool DNA Tests in Patients with Screen-Relevant Neoplasia

Incomplete marker recovery from stools, instability of long DNA, and lesion size influenced test results. Recovery of individual markers in stool when they were present in tumor tissue from the same patient was 40% (17 of 42) for SDT-1 and 39% (51 of 130) for SDT-2. The sensitivity of long DNA decreased as the time from defecation to freezing the stool increased; sensitivity for screen-relevant neoplasms decreased from 11% on stools received and frozen 12 to 24 hours after defecation to 1.8% at 24 to 35 hours and to 0% at more than 35 hours after defecation ($P = 0.010$). The sensitivity of mutation markers did not change over this time range. The median size of screen-relevant neoplasms missed by SDT-2 was 15 mm, compared with 20 mm for detected lesions ($P = 0.003$). Neither demographic variables nor randomization group affected stool DNA test positivity.

Positive Stool DNA Tests in Patients without Screen-Relevant Neoplasia

Diet and use of nonsteroidal anti-inflammatory drugs affected HemoccultSensa results but not stool DNA test or Hemoccult results. For HemoccultSensa, positivity rates in colonoscopically normal patients randomly assigned to group A (restricted diet and medications) were 2% (CI, 1% to 2%), compared with 4% (CI, 3% to 5%) for those in group B (unrestricted) ($P = 0.030$). In contrast, positivity rates for the 2 stool DNA tests were unaffected (SDT-1: 4% for group A vs. 3% for group B [$P = 0.31$]; SDT-2: 19% for group A vs. 13% for group B [$P = 0.50$]), as were those for Hemoccult (1% for group A vs. 2% for group B; $P = 0.170$). Age influenced SDT-2 results in patients with normal colonoscopy results, as positivity rates increased from 6% (CI, 4% to 29%) for patients younger than age 65 years to 26% (CI, 12% to 39%) for those 65 years of age or older ($P = 0.020$) (Table 4). Positivity rates for the component markers of SDT-2 were 6% and 15% for *APC* scanning, 0% and 8% for methylated vimentin, and 0% and 3% for mutant *K-ras* in patients younger than age 65 years and those 65 years of age or older, respectively. Other demographic factors did not affect stool DNA test positivity.

Comparison of Stool DNA Tests

We performed the 2 stool DNA tests on 69 patients with screen-relevant neoplasia and 54 with normal colonoscopy results. Positivity rates were higher with SDT-2 than with SDT-1 among the 69 patients with screen-relevant neoplasms (43% [CI, 32% to 55%] vs. 20% [11% to 30%]; $P = 0.001$), including the subset of 12 patients with colorectal cancer (58% [CI, 28% to 85%] vs. 25% [6% to 57%]; $P = 0.050$). Positivity rates were higher when comparing results for all 47 patients with adenomas 1 cm or larger (45% [CI, 31% to 59%] vs. 13% [CI, 3% to 22%]; $P = 0.003$), the 21 patients with adenomas larger than 2 cm (62% [CI, 38% to 82%] vs. 19% [CI, 5% to 42%]; $P = 0.003$), and the 26 patients with adenomas between 1 and 2 cm (31% [CI, 14% to 52%] vs. 8% [CI, 1% to 25%]; $P = 0.030$). Test positivity in those with normal colonoscopy results was significantly higher with SDT-2 than with SDT-1 (13% [CI, 5% to 25%] vs. 2% [CI, 1% to 10%]; $P = 0.030$).

Discussion

In this multicenter study, we found that the precommercial stool DNA test (SDT-1) provides no meaningful improvements over the widely used fecal occult blood tests Hemoccult and

HemoccultSensa for detection of screen-relevant colorectal neoplasms. Neoplasm detection rates by SDT-1 were no better than HemoccultSensa for any subset of neoplasms; in fact, HemoccultSensa detected significantly more cases of cancer than did SDT-1. In addition, the estimated positive likelihood ratios for cancer or high-grade dysplasia tended to be larger and the negative likelihood ratios smaller with both fecal blood tests than with SDT-1.

Because of the poor performance of SDT-1, we examined SDT-2 (which targets a potentially more informative marker panel) in a more limited manner. Stool DNA test 2 had significantly better neoplasm detection rates than did the fecal blood tests or SDT-1. We accounted for the higher detection rates of SDT-2 by better detection of advanced adenomas, a finding of particular importance for cancer prevention. Whereas fecal blood testing detected proportionately fewer proximal than distal colorectal neoplasms, corroborating previous findings (26, 27), site did not affect detection by stool DNA testing.

Detection of adenomas is essential for screening to prevent colorectal cancer. In our blinded comparison, using colonoscopy results as the criterion standard, SDT-2 detected 3 times more adenomas that were 1 cm or larger than did SDT-1, 4 times more than did Hemoccult and 3 times more than did HemoccultSensa. Compared with SDT-1, all markers in the SDT-2 panel occur early in the adenoma-to-cancer progression (25, 28, 29) and were collectively more informative for adenomas on our tissue analyses; this accounted for the superior detection rates of SDT-2 over SDT-1. Our finding that guaiac testing is insensitive for detecting adenomas is consistent with previous studies in the screening setting (21, 26, 30, 31). We did not evaluate fecal immunochemical blood tests, and such tests may detect more adenomas than do guaiac tests, because of differences in relative detection rates (32–34). Although most adenomas seem not to bleed, our data suggest that many do shed molecular markers that can be detected in stool.

Technical advances could improve DNA assay sensitivity. Although we found SDT-2 markers in tissue from nearly all screen-relevant neoplasms, the assay detected the markers in stools from the same patient in fewer than one half of cases. Methods to increase stool DNA capture are needed, and some have been reported (20, 24). Preservation of intact DNA sequences in stool could also increase marker recovery. Although long DNA was the most informative panel marker in earlier studies done with minimal processing delays (12, 15, 16), we observed that long DNA degraded during shipment to the study coordinating center and contributed little to neoplasm detection. We (35) and others (36) have shown that adding a preservative to stool at the time of defecation can prevent degradation of long DNA. Finally, because DNA markers are present in such low levels (9), augmented technical sensitivity may further enhance lesion detection (37).

Test positivity among colonoscopically normal patients was significantly more frequent with SDT-2 than with Hemoccult or HemoccultSensa, probably because of the disproportionately elevated positivity rates in older patients; however, the sample size was small and the confidence intervals were relatively wide. False-positive rates for the SDT-2, Hemoccult, and HemoccultSensa tests were similar among participants younger than 65 years of age. Other studies have reported that *K-ras* mutations (38) and methylated vimentin (20) in stool increase with age. Accumulated DNA alterations may precede endoscopically visible lesions

and account for such age-related apparent false-positives. For example, aberrant crypt foci—putative neoplasm precursors not seen by conventional colonoscopy—increase with age (39), and mutant *K-ras* (39, 40), mutant *APC* (40), and gene methylation (28, 41) have all been reported in aberrant crypt foci. Whether molecular detection of such preneoplastic field changes predicts future cancer risk is not known.

Our study differs from the only other multicenter study of stool DNA testing in average-risk patients (21) in several important ways. First, unlike the earlier report (21), we did not find that SDT-1 was better than Hemoccult for detection of invasive cancer. Second, we followed a standardized approach in a central laboratory for guaiac testing, which may account for our much higher Hemoccult sensitivity for cancer. Others have shown that central laboratory testing is more accurate than office testing after digital rectal examination (42, 43), but we do not know whether results from patient-smear stools are more accurate when processed in a centralized laboratory rather than in a physician's office. Our study also included the more sensitive HemoccultSensa test. Third, all pathology was reviewed centrally and neoplasm tissue was procured for DNA analyses, allowing us to measure marker recovery rates in the stool. Fourth, our participants were randomly assigned, which demonstrated that neither diet nor nonsteroidal anti-inflammatory drug use affected stool DNA results. Finally, the previous study did not evaluate SDT-2.

Our study provides robust data on the accuracy of guaiac-based occult blood tests in the screening setting. Published results vary widely according to testing algorithms and patient populations, and our Hemoccult results fall in reported ranges from studies done in the screening setting, as reviewed (44). Both Hemoccult and Hemoccult-Sensa achieved high specificity, regardless of diet, on tests performed according to the manufacturer's instructions and in a centralized laboratory. HemoccultSensa was more sensitive than Hemoccult for all types of screen-relevant neoplasms, and HemoccultSensa detected more than 60% of cases of cancer. However, both tests missed the large majority of the adenomas that were 1 cm or larger. Such low detection rates of premalignant adenomas may account for the reported modest (45) or absent (3, 4, 46) effect of long-term Hemoccult screening on cancer incidence.

Our study has limitations. We did not test all participants or subsets with SDT-2, and those without screen-relevant neoplasm were particularly underrepresented. We used SDT-2 to test samples from all patients with cancer, high-grade dysplasias, or adenomas larger than 2 cm; 50 randomly selected samples from patients who had adenomas measuring between 1 and 2 cm; and 75 randomly selected samples from patients with normal colonoscopy results. We did not test samples from patients with small adenomas or hyperplastic polyps by using SDT-2, and their inclusion may have further increased false-positive rates. In addition, stabilization buffer was not added to stools at time of collection. Use of a stabilization buffer may have improved detection of long DNA and possibly other DNA markers as well.

In summary, stool DNA testing by SDT-1 offered no clear advantages over fecal occult blood testing for screening detection of colorectal neoplasia. Stool DNA test 2, which targets more broadly informative markers, detected significantly more screen-relevant neoplasms than did Hemoccult or HemoccultSensa, and detection was especially improved for

adenomas. However, we do not know whether this gain in sensitivity is offset by a loss in specificity. Molecular technology will continue to evolve and provide opportunities for needed assay refinements in stool DNA testing.

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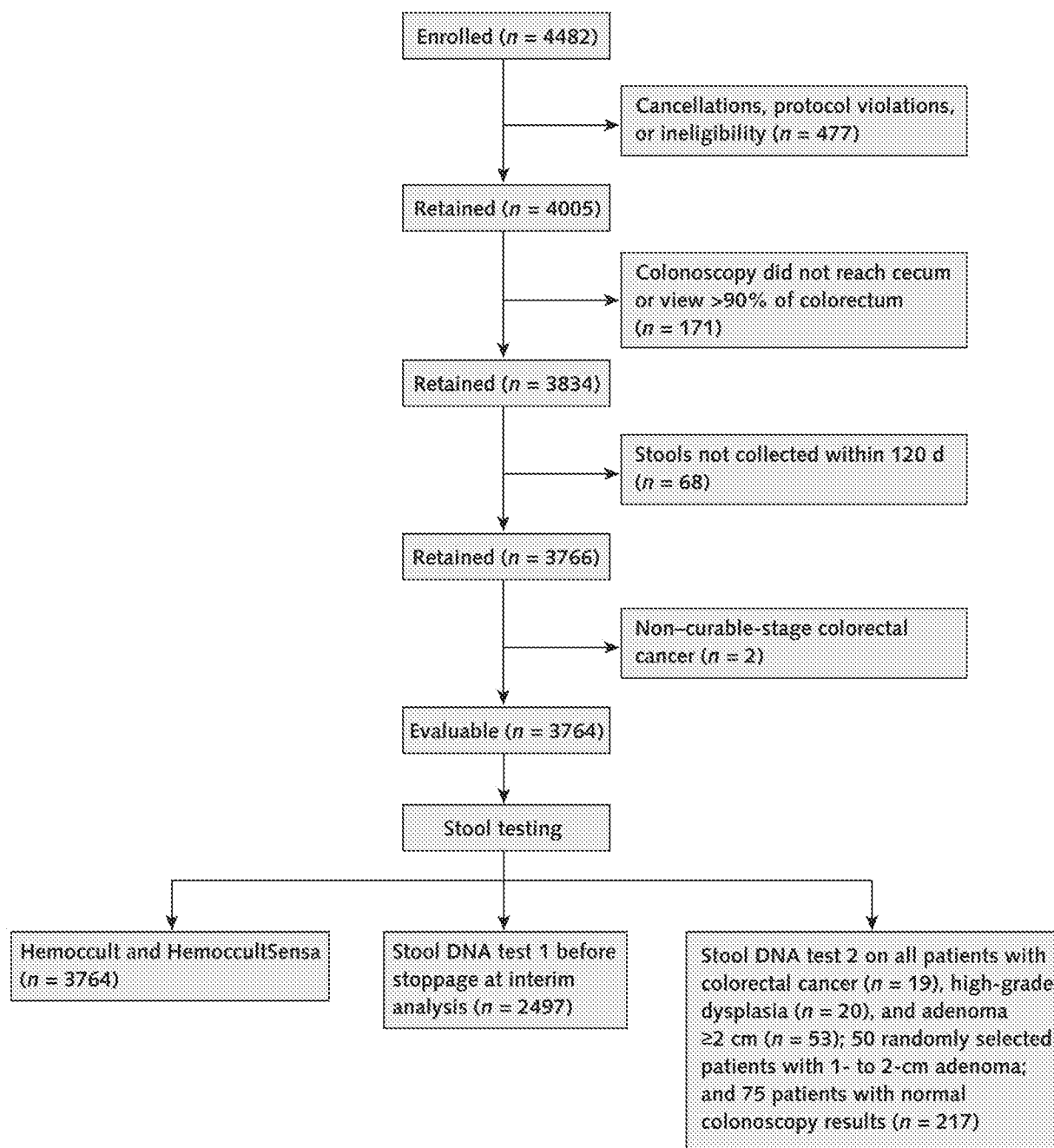


Figure 1. Study flow diagram

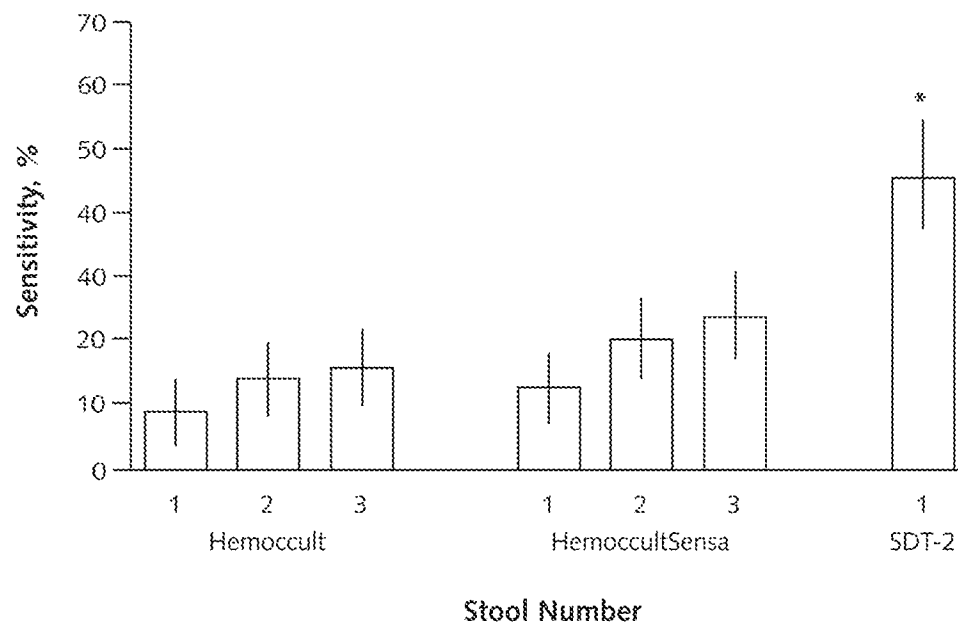


Figure 2. Stool DNA versus occult blood testing for detection of screen-relevant neoplasia (curable-stage colorectal cancer, high-grade dysplasia, and adenomas ≥ 1 cm) ($n = 142$)
Sensitivity is plotted for Hemocult and HemocultSensa with 1, 2, and 3 stools per patient and with stool DNA test 2 (SDT-2) on a single stool per patient. Vertical lines represent 95% CIs.

* $P < 0.001$ versus Hemocult or HemocultSensa.

Table 1

Definitions

Gene targets in stool DNA test panels:

Test 1: point mutations on *K-ras*, *APC*, and *p53*, microsatellite marker BAT-26; long DNA

Test 2: point mutations on *K-ras*, scanned mutator cluster region of *APC*, vimentin methylation

Screen-relevant neoplasia: colorectal cancer, high-grade dysplasia, adenomas ≥ 1 cm

Sensitivity: rate of test positivity for those with screen-relevant neoplasia

Specificity: rate of test negativity for those without screen-relevant neoplasia

Test positivity: rate of positive stool test results for individual colonoscopic findings or groups of findings

Table 2

Baseline Characteristics and Colorectal Findings

Characteristic	All Patients (n = 4482)	Evaluable Patients [*] (n = 3764)	Patients Tested with SBT-1 [†] (n = 2497)	Patients Tested with SBT-2 [‡] (n = 217)
Age, y				
Mean (SD)	63.8 (8.29)	63.7 (8.25)	60.4 (7.86)	66.4 (7.17)
Median (range)	65 (50–81)	65 (50–80)	59 (50–80)	67 (51–80)
Women, n (%)	2341 (52.2)	1964 (52.2)	1348 (54.0)	108 (49.8)
White, n (%)	4184 (93.4)	3522 (93.6)	2314 (92.7)	201 (92.6)
Colorectal findings, n (%)				
Screen-relevant neoplasia	—	290 (7.7)	157 (6.3)	142 (65.4)
Cancer				
Stage I	—	11 (0.3)	6 (0.2)	11 (5.1)
Stages II and III	—	8 (0.2)	6 (0.2)	8 (3.7)
Cancer + high-grade dysplasia	—	39 (1.0)	22 (0.9)	39 (18.0)
Adenoma ≥1 cm	—	251 (6.7)	135 (5.4)	103 (47.5)
Adenoma >2 cm	—	53 (1.4)	21 (0.8)	53 (24.4)
Adenoma <1 cm	—	785 (20.9)	469 (18.8)	Not tested
Hyperplastic polyps	—	492 (13.1)	341 (13.7)	Not tested
Other	—	86 (2.3)	57 (2.3)	Not tested
Normal	—	2111 (56.1)	1473 (59.0)	75 (34.6)

SDT = stool DNA test.

* Patients who met all inclusion criteria. Both Hemocult and HemocultSensa (Beckman Coulter, Fullerton, California) were performed on all evaluable participants.

[†] On the basis of results from an interim analysis, SDT-1 was terminated.

[‡] All participants with cancer, high-grade dysplasia, and adenomas ≥2 cm from the full enrollment period are included, as are random samples from 50 patients with 1- or 2-cm adenomas and 75 with normal colonoscopy results.

Table 3

Summary of Test Performance

Index Test	Screen-Relevant Neoplasia, [*] <i>n</i>	Positive Test Result, <i>n</i>	Sensitivity (95% CI)	No Screen- Relevant Neoplasia, <i>n</i>	Negative Test Result, <i>n</i>	Specificity (95% CI)	Positive Likelihood Ratio (95% CI)	Negative Likelihood Ratio (95% CI)
Hemoccult (<i>n</i> = 2497)	157	17	11 (6–16) [†]	2340	2297	98 (98–99) [‡]	5.9 (3–10)	0.9 (0.9–1.0)
HemoccultSensa (<i>n</i> = 2497)	157	33	21 (15–27) [§]	2340	2258	97 (96–97)	6.0 (4–9)	0.8 (0.8–0.9)
SDT-1 (<i>n</i> = 2497)	157	31	20 (14–26)	2340	2246	96 (95–97)	4.9 (3–7)	0.8 (0.8–0.9)
SDT-2 (<i>n</i> = 217)	142	66	40 (32–49) [¶]	75	NA ^{**}	NA	NA	NA

NA = not available; SDT = stool DNA test.

^{*} Includes curable-stage cancer, high-grade dysplasia, and adenomas ≥ 1 cm.

[†] $P = 0.02$ for STD-1 vs. Hemoccult.

[‡] $P < 0.001$ for STD-1 vs. Hemoccult.

[§] $P = 0.80$ for STD-1 vs. HemoccultSensa.

^{||} $P = 0.40$ for STD-1 vs. HemoccultSensa.

[¶] We calculated the weighted sensitivity for SDT-2 with the following equation:

$$\text{reweighted sensitivity} = (\% [\text{colorectal cancer} + \text{high-grade dysplasia}] \times \text{PR}) + (\% \text{ adenomas} \geq 2 \text{ cm} \times \text{PR}) + (\% \text{ adenomas} < 2 \text{ cm} \times \text{PR}) \\ = (0.13 \times 0.49) + (0.18 \times 0.57) + (0.68 \times 0.34)$$

PR = proportion of participants for that category of screen-relevant neoplasia in the entire population with screen-relevant neoplasia. See "Comparison of Stool DNA Tests" for statistical comparisons of SDT-1 and SDT-2 in participants who had both DNA tests performed.

^{**} We did not calculate formal specificity because SDT-2 was not performed on all subsets without screen-relevant neoplasia.

Table 4

Test Positivity Rates

Stool DNA Test and Patient Subset	Positive SDT Result (95% CI), %	Positive Hemocult Result (95% CI), %	P Value ^a	Positive HemocultSensa Result (95% CI), %	P Value ^b
SDT-1					
Screen-relevant neoplasms ^c (n = 157)	20 (14–26)	11 (6–16)	0.020	21 (15–27)	0.80
Site					
Proximal ^d (n = 56)	20 (9–30)	7 (2–17)	0.070	11 (3–19)	0.20
Distal ^d (n = 101)	20 (12–28)	13 (6–19)	0.100	27 (18–35)	0.20
Cancer (n = 12)	25 (5–57)	50 (22–78)	0.30	75 (51–100)	0.019
Cancer + high-grade dysplasia (n = 22)	36 (16–56)	41 (20–61)	0.80	55 (34–75)	0.20
Adenoma ≥1 cm ^e (n = 135)	17 (11–23)	6 (2–10)	0.004	16 (9–22)	0.74
Adenoma >2 cm (n = 21)	19 (5–42)	14 (3–36) ⁱ	0.60	33 (13–54)	0.30
Adenoma 1–2 cm (n = 114)	17 (10–24)	4 (1–8) ⁱⁱⁱ	0.004	12 (6–18)	0.40
Adenoma <1 cm (n = 469)	4 (2–6)	3 (1–5)	0.30	5 (3–7)	0.70
Hyperplastic polyps (n = 341)	5 (2–7)	1 (0.2–3) ⁱⁱⁱ	0.003	4 (2–6)	0.70
Other (n = 57)	5 (1–15)	2 (0.04–9)	0.30	5 (1–15) ^d	1.00
Normal (n = 1473)	4 (3–5)	2 (1–2)	<0.001	3 (2–4)	0.20
Age <65 y (n = 1040)	3 (2–4)	2 (1–2)	0.009	3 (2–4)	0.40
Age ≥65 y (n = 433)	5 (3–7)	2 (1–3)	0.020	3 (2–5)	0.30
SDT-2					
Screen-relevant neoplasms ^c (n = 142)	46 (38–55)	16 (10–22)	<0.001	24 (17–31)	<0.001
Site					

Stool DNA Test and Patient Subset	Positive SDT Result (95% CI), %	Positive Hemocult Result (95% CI), %	P Value [*]	Positive HemocultSensa Result (95% CI), %	P Value [†]
Proximal [§] (n = 56)	45 (32–58)	9 (1–16)	<0.001	13 (4–21)	<0.001
Distal [§] (n = 86)	48 (37–58)	21 (12–30)	<0.001	31 (22–41)	0.030
Cancer (n = 19)	58 (36–80)	47 (25–70)	0.40	63 (41–85)	0.70
Cancer + high-grade dysplasia (n = 39)	49 (33–64)	33 (19–48)	0.100	44 (28–59)	0.60
Adenoma ≥ 1 cm [‡] (n = 103)	46 (35–54)	10 (4–15)	<0.001	17 (9–24)	<0.001
Adenoma > 2 cm (n = 53)	57 (43–70)	13 (4–22)	<0.001	25 (13–36)	<0.001
Adenoma 1–2 cm (n = 50)	34 (21–47)	6 (1–17)	0.002	8 (2–19)	0.005
Normal (n = 75)	16 (8–24)	4 (1–11)	0.010	5 (1–13)	0.030
Age <65 y (n = 36)	6 (4–29)	3 (0–15)	0.56	6 (1–19)	1.00
Age ≥65 y (n = 39)	26 (12–39)	5 (1–17)	0.010	5 (1–17)	0.010

SDT = stool DNA test.

^{*} For SDT versus Hemocult.

[†] For SDT versus HemocultSensa.

[‡] Patients were classified by the most advanced lesion found (in descending order of severity: cancer, high-grade dysplasia, or adenoma). The distribution of screen-relevant neoplasms differs from that in Table 3. See Table 2 for details of patients tested with SDT-2.

[§] Proximal and distal sites are relative to the splenic flexure. "Proximal" includes the cecum and ascending and transverse colon; "distal" includes the splenic flexure, descending colon, sigmoid, and rectum.

^{||} The exact CI was used when the numerator was <5.

Table 5

Presence of DNA Markers in Tumor Tissue^{*}

Marker	SDT-1 Panel						SDT-2 Panel				
	Patients, <i>n</i>	<i>K-ras</i> , %	<i>APC</i> [†] , %	<i>p53</i> , %	BAT-26, %	Full Panel, %	Patients, <i>n</i>	<i>K-ras</i> , %	<i>APC</i> [‡] , %	Vimentin, %	Full Panel, %
Cancer and high-grade dysplasia	20	45	35	25	0	60	35	51	60	63	94
Adenoma ≥1 cm	48	42	38	6	2	63	99	39	73	63	98
All screen-relevant neoplasms [§]	68	43	37	12	1	62	134	43	69	63	97

SDT = stool DNA test.

^{*} Tissues were analyzed from all available samples from patients with cancer and high-grade dysplasia and from a random sample of patients with adenomas ≥1 cm.

[†] The assay targeted 10 high-frequency mutations on the *APC* gene by using a single-base extension method (see citations in Methods).

[‡] The assay targeted any mutation in the mutator cluster region of the *APC* gene by using the scanning method (see citations in Methods).

[§] Includes curable-stage cancer, high-grade dysplasia, and adenomas ≥1 cm.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: *Ex Parte* Reexamination of U.S. Patent No. 11,634,781
Issued: April 25, 2023
App. Ser. No.: 17/936,335
Filing Date: September 28, 2022
Named Inventor: Joost Louwagie
Title: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING
DETECTION OF BLOOD

Mail Stop: *Ex Parte* Reexam
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MR. ANTHONY P. SHUBER, MS

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I, Mr. Anthony P. Shuber, MS, declare and state as follows:

1. I am submitting this declaration in connection with a Request for *Ex Parte* Reexamination of U.S. Patent No. 11,634,781 (“the ‘781 Patent”). I have been asked by the Requester to provide information and opinions regarding the level of skill of a person of ordinary skill in the art for the invention claimed in the ‘781 Patent, the general knowledge of such a person at the time of the effective filing date for the ‘781 Patent, the scope and content of the prior art references, whether the invention claimed in the ‘781 Patent was previously described by the prior art, and whether the invention claimed in the ‘781 Patent would have been obvious to a person of ordinary skill in the art in view of the prior art.

2. For my work on this case I am charging my customary consulting rate. My opinions and work on this matter are not dependent on the outcome of this matter.

I. RELEVANT EXPERIENCE

3. As described in my *curriculum vitae*, which is attached hereto, my research interests include developing Molecular Diagnostic Assays applied to both inherited diseases and cancer.

4. In 1980, I received a Bachelor of Science degree in Biology from Marquette University in Milwaukee, Wisconsin. Thereafter, in 1983, I received a Master of Science degree in Molecular and Developmental Biology from Marquette University in Milwaukee, Wisconsin.

5. My prior work in the field includes developing several DNA diagnostics tests, while employed at Genzyme Genetics. One of those tests involved a novel and proprietary approach to multi-plex and analyze hundreds of inherited disease diagnostic mutations that spanned across multiple diseases such as Cystic Fibrosis, Sickle Cell Anemia, and several others. Subsequent to my work in developing molecular diagnostic assays for inherited disorders, I focused the majority of my efforts on developing diagnostics in oncology, with my major area of interest involving the early detection of cancer.

6. Currently, I am employed as Chief Innovation Officer at Harbinger Health.

7. My research interests include developing and creating novel diagnostic assays for the early detection of cancer as well as novel approaches to cancer treatment. In my career, I authored or co-authored over 35 journal articles on diagnostics methodologies that have been

published in peer-reviewed journals. Furthermore, I am a named inventor or co-inventor on at least 100 patents or patent applications.

II. DOCUMENTS AND INFORMATION CONSIDERED

8. In providing the information and opinions in this declaration, I have reviewed and relied upon the documents listed below, each of the other documents referred to in this declaration, and the documents that are attached as exhibits to this declaration.

Ex. No.	Prior Art Citation
1001	U.S. Patent No. 11,634,781 (“the ‘781 Patent”)
1002	U.S. Patent No. 5,952,178 to Lapidus et al. (“Lapidus ‘178”)
1003	U.S. Patent No. 5,741,650 to Lapidus et al. (“Lapidus ‘650”)
1004	Ahlquist et al., “Stool DNA and Occult Blood Testing for Screen Detection of Colorectal Neoplasia,” <i>Ann Intern Med.</i> , 149(7):441-W81 (published in final form October 7, 2008) (“Ahlquist”)
1005	Patent Cooperation Treaty (PCT) Publication No. WO 2005/014154 to O’Neill (“O’Neill”)
1006	European Patent Application Publication No. EP1366715A1 to De Luca et al. (“De Luca”)
1007	Olson et al., “DNA Stabilization Is Critical for Maximizing Performance of Fecal DNA-Based Colorectal Cancer Tests,” <i>Diagn Mol Pathol</i> , 14:183–191 (published September 2005) (“Olson”)
1008	Leung et al., “Detection of Hypermethylated DNA or Cyclooxygenase-2 Messenger RNA in Fecal Samples of Patients With Colorectal Cancer or Polyps,” <i>Am J Gastroenterol</i> , 102:1070–1076 (2007) (“Leung”)
1009	Hoepffner et al., “Comparative evaluation of a new bedside faecal occult blood test in a prospective multicentre study,” <i>Aliment Pharmacol Ther</i> , 23, 145–154 (published 2006) (“Hoepffner”)
1010	U.S. Patent No. 4,859,610 to Maggio (“Maggio”)
1011	U.S. Patent No. 5,196,167 to Guadagno et al. (“Guadagno”)
1012	U.S. Patent No. 5,137,806 to LeMaistre et al. (“LeMaistre”)
1013	U.S. Patent No. 5,527,676 to Vogelstein et al. (“Vogelstein”)

9. In addition to the materials above, I relied upon my education and experience, as detailed above and in my attached *curriculum vitae*, to provide the information and opinions in this declaration.

III. APPLICABLE LEGAL STANDARDS

10. I understand that the issues presented in this *ex parte* reexamination must be considered in view of certain applicable legal standards. I am not a lawyer. However, the following is a summary of my general understanding of certain legal principles and standards that I have used in forming my opinions expressed below, including, in particular, my general understanding of the legal concepts of “anticipation” and “obviousness.”

11. I understand that 35 U.S.C. § 102 (“§ 102”) provides the standard for determining whether a claim in a patent is invalid as anticipated (“not novel”). I understand that a claim in a patent is invalid as anticipated under §102 when each and every element of the claim is described in a single prior art reference as understood by a person of ordinary skill in the art. I understand that the only grounds for anticipation that may be presented in a request for *ex partes* reexamination are those based on patents and/or printed publications. I further understand that for *ex parte* reexamination, anticipation must be proven by a preponderance of the evidence, which I understand to mean “more likely than not.”

12. I understand that 35 U.S.C. § 103 (“§ 103”) provides the standard for determining whether a claim in a patent is invalid as obvious. I understand that a claim in a patent is invalid as obvious under § 103 when the differences between the claimed subject matter and the prior art are such that the claimed subject matter as a whole would have been obvious at the time the invention was made to a person of ordinary skill in the art. I understand that the only grounds for obviousness that may be presented in a request for *ex partes* reexamination are those based on patents and/or printed publications. I further understand that for *ex partes* reexamination, obviousness must be proven by a preponderance of the evidence, which I understand to mean “more likely than not.”

13. I understand that, in conducting the obviousness analysis, it is important to identify a reason that would have prompted a person of ordinary skill in the art to combine prior art references in a way that would have rendered the claimed invention obvious and that the person would have had a reasonable expectation of success in making the claimed invention. I

also understand that whether or not it would have been obvious to combine or modify prior art may depend in part on whether such a person would have found the claimed invention to be a predictable use of prior art elements according to their established functions.

14. I understand that a prior art reference may be considered in assessing a patent claim's obviousness if the reference discloses solving any problem or addressing any need addressed by the patent, or if the reference discloses information having obvious uses beyond its primary purpose that a person of ordinary skill in the art would reasonably examine to solve a problem or address a need presented by the patent. I understand that a claimed combination of familiar elements using known methods is likely obvious when it does no more than yield predictable results. I understand that when a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same or a different field. I understand that if a person of ordinary skill in the art can implement a predictable variation of a claimed invention, then the claimed invention is likely obvious.

15. I understand that an obviousness analysis need not seek out precise teachings directed to the specific subject matter of the claimed invention, and that the Patent Office can take account of the inferences and creative steps that a person of ordinary skill in the art would have employed at the time of the alleged invention. I understand that it is often necessary to look to interrelated teachings of multiple prior art references; the effects of demands known to those working in the relevant field of art or present in the marketplace; and the background knowledge possessed by a person of ordinary skill in the art, all in order to determine whether there was an apparent reason for the person of ordinary skill in the art to have combined elements in the manner claimed. I understand that any need or problem known in the field of endeavor at the time of the alleged invention or addressed by the patent-at-issue can provide a reason for the person of ordinary skill in the art to have combined elements in the manner claimed. I understand that one way in which a patent's subject matter can be proved obvious is by noting that there existed at the time of invention a known problem for which there was an obvious solution encompassed by the patent's claims. I understand that when there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. I further understand that, if this leads to the anticipated success, it is likely the result not of innovation but of ordinary skill and common sense and, as such, likely is obvious.

16. I also understand that in determining the meaning of claim terms in a patent, the claim terms of patents are given the broadest reasonable interpretation consistent with the specification and that limitations in the specification are not read into the claims.

17. Detailed explanations of my opinions in this matter as well as the basis for each of my opinions are set forth below. I reserve the right to supplement my opinions based on any new information that is provided to me in this matter.

IV. QUALIFICATIONS AND KNOWLEDGE OF A PERSON OF ORDINARY SKILL IN THE ART

18. It is my opinion that the relevant field of the invention of the '781 Patent is the field of processing and evaluating fecal samples.

19. I understand that the knowledge level of one with ordinary skill in the art is determined by considering various factors, including the type of problems encountered in the art; prior art solutions to those problems; rapidity with which innovations are made; sophistication of the technology; and educational level of active workers in the field. I understand that the earliest possible effective filing date that could be accorded the '781 Patent is February 3, 2009. Solely for the purpose of this declaration, I have been asked to assume that February 3, 2009, is the effective filing date of each of the claims of the '781 Patent and to address the ordinary level of skill in the art at that time.

20. Taking into account the above factors, and based on my experience in this field and with persons working in this field, it is my opinion that a person of ordinary skill in the art of the '781 Patent as of February 3, 2009, would have had at least a Bachelor of Science degree in Biology (or a similar major) and several years of experience in the processing of biological samples, such as fecal samples from a human. Such a person would have been well-acquainted with methods of collecting fecal samples, preserving proteins and/or nucleic acids, including hemoglobin, DNA, and RNA, and subsequently evaluating the fecal samples for proteins and/or nucleic acids, including hemoglobin, DNA, and RNA, as of February 3, 2009.

21. I currently possess the level of ordinary skill in the art of the '781 Patent. I also possessed that level of skill as of February 3, 2009. Furthermore, I possessed that level of skill at the time I co-invented subject matter described and claimed in the Lapidus '178 and Lapidus

'650 references (further discussed below). All of my opinions in this declaration are from the perspective of a person of ordinary skill in this art of the '781 Patent as of February 3, 2009.

V. SUMMARY OF THE '781 PATENT

22. I have reviewed the claims and specification for the '781 Patent and provide the following summary of the claimed invention of the '781 Patent. A copy of the '781 Patent is attached to the Request as Exhibit 1001.

23. The '781 Patent generally describes methods of processing a fecal sample from a human subject. Ex. 1001 at Abstract. The methods of the '781 Patent involve collecting a fecal sample, removing a portion of a collected fecal sample and combining the removed portion with a buffer that prevents denaturation or degradation of blood proteins, and combining a buffer with the remaining portion of the fecal sample. *Id.*

24. The '781 Patent includes one independent claim as explained below.

VI. CLAIM TERMS IN THE '781 PATENT

25. I have reviewed the claims and specification for the '781 Patent and provide the following comments on certain terms found in the claims of the '781 Patent. A copy of the '781 Patent is attached to the Request as Exhibit 1001.

26. My opinions regarding the meaning of claim terms of the '781 Patent are based on the plain and ordinary meanings of the claim terms in the context of the entire '781 Patent, including its specification and prosecution history.

27. The claims of the '781 Patent require that a fecal sample is processed "without freezing." The ordinary meaning of "freezing" is being at or below the temperature at which water freezes and the temperature at which water freezes is known to be 0°C (32°F). Thus, a person of ordinary skill in the art would understand "without freezing" to include any temperature above 0°C (32°F) or a temperature range that includes a temperature above 0°C (32°F).

28. The claims of the '781 Patent require that a fecal sample is collected "at home." The '781 Patent uses this term primarily to distinguish the location of collection from the testing facility. For instance, the '781 Patent states that "[f]ollowing collection of the sample and suitable processing, the method may further comprise forwarding or otherwise delivering the

removed portion of the collected faecal sample to a laboratory for performing step (a) of the method, as defined herein, on the removed portion of the collected faecal sample.” *Id.* at column 18, lines 32-35 (emphasis added). Similarly, dependent claim 2 of the ‘781 Patent indicates that the method of claim 1 further comprises “delivering the [fecal sample collected at home] to a medical diagnostics laboratory.” *Id.* at column 45, lines 39-44. Therefore, a person of ordinary skill in the art would understand “at home,” as used in the ‘781 Patent, to refer to a location other than the testing facility.

29. The ‘781 Patent defines “epigenetic modification” such that it includes “any alteration in the DNA, generally resulting in diminished gene expression, which is mediated by mechanisms other than alterations in the primary nucleotide sequence of a gene.” *Id.* at column 12, lines 35-39. A skilled person would understand the ‘781 Patent’s definition of “epigenetic modification” as the plain and ordinary meaning of the term.

VII. COMPARISON OF CLAIMS OF THE ‘781 PATENT WITH PRIOR ART REFERENCES

30. I have been asked to review various prior art references that relate to the claimed invention of the ‘781 Patent, including Lapidus ‘178 and Lapidus ‘650. I am one of the named inventors of both Lapidus ‘178 and Lapidus ‘650 and, thus, am very familiar with their contents.

31. I understand that Lapidus ‘178 is prior art to the ‘781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was patented on September 14, 1999, more than one year before the earliest possible effective filing date for the claims of the ‘781 Patent (February 3, 2009).

32. I understand that Lapidus ‘650 is prior art to the ‘781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was patented on April 21, 1998, more than one year before the earliest possible effective filing date for the claims of the ‘781 Patent (February 3, 2009).

33. As discussed in detail below, it is my opinion that the description of Lapidus ‘178 and the description of Lapidus ‘650 each anticipate claims 1-4 and 12 of the ‘781 Patent and also render claims 1-4 and 12 of the ‘781 Patent obvious.

34. I have also reviewed and relied on Maggio as evidencing certain aspects of the disclosure of Lapidus ‘178 and Lapidus ‘650. I understand that Maggio is prior art to the ‘781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was patented on August 22, 1989, more than

one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

35. I have also reviewed and relied upon the disclosures of Guadagno, LeMaistre, and Vogelstein, each of which are incorporated by referenced into both Lapidus '178 and Lapidus '650. Furthermore, I understand that Guadagno, LeMaistre, and Vogelstein are also themselves prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(b) because they were each patented more than one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

36. I also reviewed the disclosures of Ahlquist, O'Neill, De Luca, Olson, Leung, and Hoepffner.

37. I understand that Ahlquist is prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(a) because it was published on October 7, 2008, and reasonably accessible to the public interested in the art before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

38. I understand that O'Neill is prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was published on February 17, 2005, more than one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

39. I understand that De Luca is prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was published on December 3, 2003, more than one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

40. I understand that Olson is prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was published in September 2005, more than one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

41. I understand that Leung is prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was published in 2007, and reasonably accessible to the public interested in the art more than one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

42. I understand that Hoepffner is prior art to the '781 Patent under pre-AIA 35 U.S.C. § 102(b) because it was published in 2006, and reasonably accessible to the public interested in the art more than one year before the earliest possible effective filing date for the claims of the '781 Patent (February 3, 2009).

43. As discussed in detail below, it is my opinion that the teachings of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill, in combination with the teachings of one another and/or with De Luca, Olson, Leung, and/or Hoepffner, render claims 1-20 of the '781 Patent obvious. It is also my opinion, discussed below, that a person of ordinary skill in the art as of February 3, 2009 would have been motivated to combine Lapidus '178, Lapidus '650, Ahlquist, O'Neill, De Luca, Olson, Leung, and/or Hoepffner with a reasonable expectation of success.

A. Claim 1 Was Anticipated and Obvious

1) Preamble of Claim 1

44. The preamble of claim 1 of the '781 Patent provides "[a] method of processing a freshly-collected fecal sample without freezing, the method comprising...". As stated above, a person of ordinary skill in the art would understand that "without freezing" includes any temperature above 0°C (32°F), or a temperature range that includes a temperature above 0°C (32°F). Lapidus '178, Lapidus '650, and Ahlquist all teach methods of processing freshly-collected fecal samples without freezing.

45. Lapidus '178 describes a freshly-collected stool (a fecal sample) voided by a patient into a receptacle that is transported to a testing facility. Ex. 1002 at column 7, lines 58-64. Lapidus '178 states that, in "a preferred embodiment, a stool is frozen prior to processing." *Id.* at column 9, lines 21-22. This statement reflects that the inventors (Mr. Lapidus and I) also contemplated non-preferred embodiments in which the stool was not frozen prior to processing. As such, Lapidus '178 discloses this feature of claim 1.

46. Lapidus '650 teaches processing of freshly-collected stool samples and does not teach freezing those samples. Ex. 1003 at column 7, lines 12-17; *id.* at column 7, lines 24-26. Additionally, Lapidus '650 (at column 6, lines 30-31) incorporates by reference U.S. Patent No. 5,196,167 ("Guadagno"), which discloses a test sheet product used for identification of blood in a patient stool and states that "[a]fter the patient has completed a bowel movement, the test sheet is merely dropped into the toilet bowl." Ex. 1011 at column 6, lines 65-67. Guadagno further discloses that "[a]fter the results of the test have been visually observed, the test sheet is disposed of by merely flushing the toilet bowl." *Id.* at column 7, lines 7-9. Accordingly, a person of ordinary skill in the art would recognize Lapidus '650 discloses the method of processing a "freshly-collected" fecal sample "without freezing."

47. Furthermore, any minor differences that may exist between the preamble of claim 1 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would be used to process freshly-collected fecal samples without freezing those samples. Thus, at a minimum, this aspect of claim 1 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

48. Ahlquist also teaches methods of processing freshly-collected fecal samples without freezing. Ex. 1004 at page 4, at 'Stool Collection and Processing' heading. Ahlquist discloses "[p]atients collected 3 stools by using plastic buckets mounted to the toilet seat. Promptly after each individual collection, patients smeared stool onto both windows of their Hemocult and HemocultSensa cards and then express-shipped smeared cards and the whole stool (sealed in a bucket in an insulated container cooled with ice packs) to the Mayo Clinic in Rochester, Minnesota. *Id.* Further, Ahlquist indicates that the stools were frozen after they were received at the Mayo Clinic. *Id.* This indicates to a person skilled in the art that the freshly-collected fecal samples were processed by the patients without freezing in Ahlquist. As such, Ahlquist teaches the preamble of claim 1.

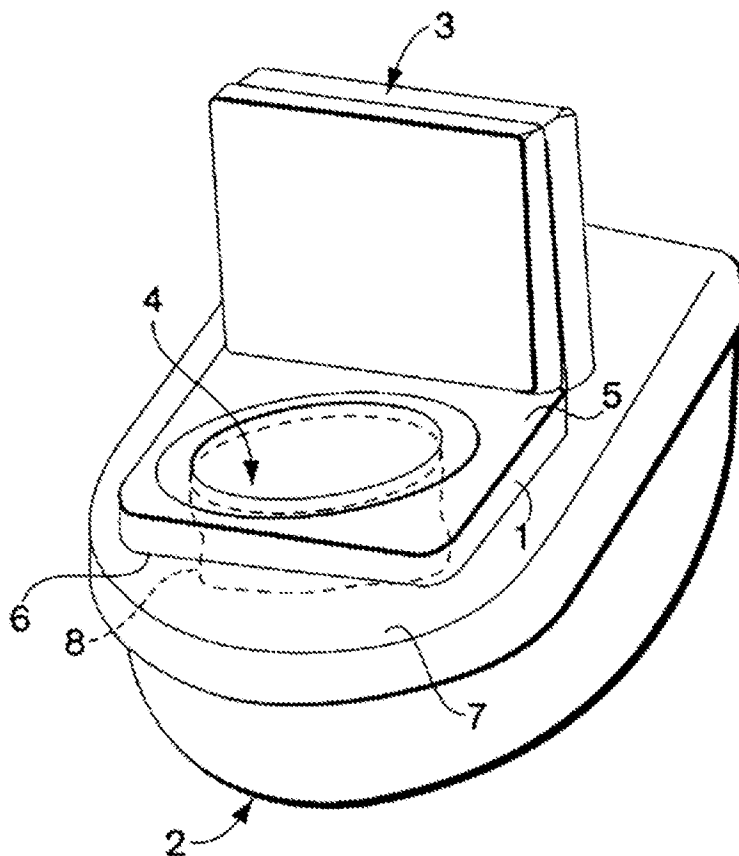
49. O'Neill also teaches methods of processing freshly-collected fecal samples without freezing. Ex. 1005 at page 6, lines 14 to page 8, line 11. O'Neill discloses collection of stool (a fecal sample) into a container. *Id.* Although O'Neill suggests that the stools can be cooled to a temperature below its "gel freezing" point, O'Neill teaches that this temperature range includes temperatures as high as 15°C (59°F). *Id.* at page 6, lines 14-17. A person of ordinary skill in the art would understand that collection of a fecal sample at a temperature of 15°C (59°F) corresponds to processing the sample "without freezing." As such, O'Neill teaches the preamble of claim 1.

2) First Limitation of Claim 1

50. The first limitation of claim 1 of the '781 Patent provides "a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject by defecation directly into a sealable collection vessel...". As stated above, a person of ordinary skill in the art would have understood "at home", as used in the '781 Patent, to refer to a location other than the testing facility (e.g., a medical diagnostics laboratory).

51. Lapidus '178 and Lapidus '650 each teach collecting a fecal sample from a human subject at home by defecation directly into a sealable collection vessel. For instance, Lapidus '178 and '650 both describe that “[t]he receptacle may be fitted to a conventional toilet such that the receptacle accepts stool voided in a conventional manner.” Ex. 1002 at column 7, lines 64-66; Ex. 1003 at column 7, lines 26-28. This passage provides evidence that the fecal sample is collected from a human subject, since only humans use conventional toilets to void stool in a conventional manner.

52. Furthermore, Lapidus '178 and Lapidus '650 both describe that the fecal sample is collected in a receptacle that can be adapted to fit a toilet, thus demonstrating that the human subject defecates “directly” into the receptacle. As shown in Figure 2 of those references, the receptacle is a “sealable” receptacle comprising an articulating cover 3 that can be utilized in “a closed position (not shown) for sealing voided stool within the receptacle.” Ex. 1002 at column 8, lines 24-27; Ex. 1003 at column 7, lines 54-55. Figure 2 is reproduced below:



53. Additionally, Lapidus '178 and Lapidus '650 both describe that the stool can be collected at home. As discussed above, a person of ordinary skill in the art would interpret “at

home,” as that term is used in the ‘781 Patent, to include any location other than a testing facility. Lapidus ‘178 and Lapidus ‘650 both describe that the sealable receptacle containing the fecal sample “is preferably small enough to be transported to a testing facility” (Ex. 1002 at column 7, lines 63-64; Ex. 1003 at column 7, lines 24-26), meaning that the fecal sample is collected at *a location different than the testing facility*. Further, Lapidus ‘178 and Lapidus ‘650 both describe that the toilet onto which the receptacle may be fitted is “a conventional toilet such that the receptacle accepts stool voided in a conventional manner.” Ex. 1002 at column 7, lines 62-64; Ex. 1003 at column 7, lines 26-28.) This indicates to persons skilled in the art that a fecal sample is collected at home by the patient since the “conventional manner” for voiding stool is on a toilet in one’s home. In sum, both Lapidus ‘178 and Lapidus ‘650 disclose the first limitation of claim 1.

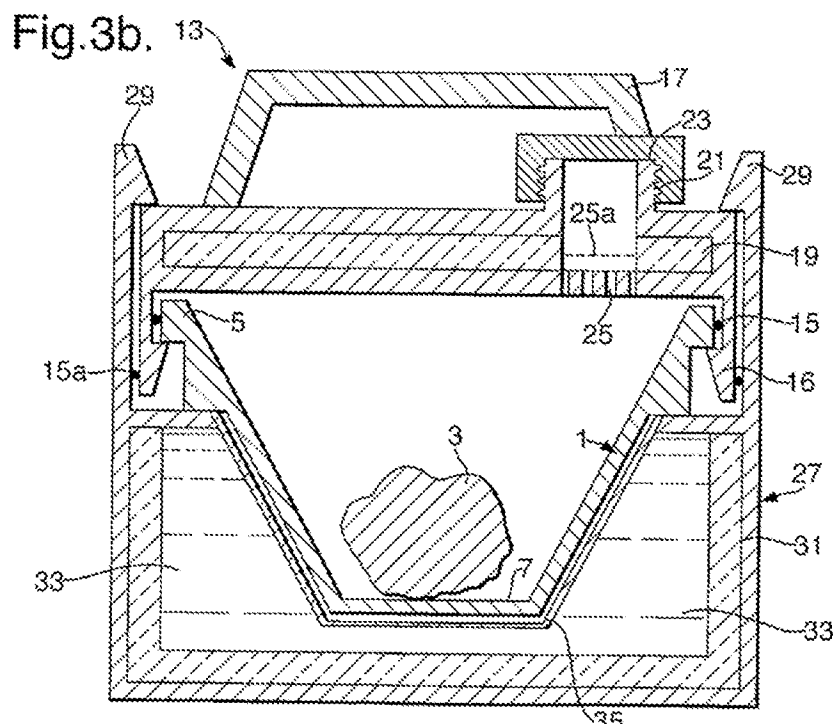
54. Furthermore, any minor differences that may exist between the first limitation of claim 1 and the teachings of Lapidus ‘178 and Lapidus ‘650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus ‘178 and Lapidus ‘650 patents, I envisioned that the methods described in those references could and would involve collecting a fecal sample from a human subject at home by defecation directly into a sealable collection vessel. Thus, at a minimum, this aspect of claim 1 was obvious in view of the teachings of Lapidus ‘178 and/or Lapidus ‘650.

55. Ahlquist also teaches collecting a fecal sample from a human subject at home by defecation directly into a sealable collection vessel. Ahlquist discloses that human patients collected stools (a fecal sample) into a plastic bucket mounted to a toilet seat. Ex. 1004 at page 4, at ‘Stool Collection and Processing’ heading. This indicates to a person skilled in the art that the human subjects in Ahlquist defecated directly into the plastic bucket, which corresponds to the “collection vessel” of claim 1. Further, Ahlquist teaches that the plastic bucket is a sealable collection vessel because the plastic bucket is later sealed upon shipping. *Id.*

56. Additionally, Ahlquist describes that the stool can be collected at home. As discussed above, a person of ordinary skill in the art would interpret “at home,” as that term is used in the ‘781 Patent, to include any location other than a testing facility. Ahlquist discloses that the stools are collected by patients and then express-shipped to the Mayo Clinic, meaning that the fecal sample was collected *at a location different than the Mayo Clinic testing facility*.

This indicates that the stool can be collected in a toilet that is not located at a testing facility. In sum, Ahlquist discloses the first limitation of claim 1.

57. O'Neill also teaches collecting a fecal sample from a human subject at home by defecation directly into a sealable collection vessel. O'Neill discloses that human patients collected stools (a fecal sample) into a container positioned over a toilet. Ex. 1005 at page 8, lines 24-30 ("The container may further have installation means (which may be detachable from the container) for removably installing the container on a toilet (e.g. below the toilet seat) with the receiving mouth opening upwardly and the collection channel extending horizontally beneath the mouth. This allows the user to produce the stool in the usual manner, which promotes user acceptability."). O'Neill teaches that the container is "the receptacle into which the stool is excreted." *Id.* at page 7, line 34- page 8, line 11. Figure 3b of O'Neill is presented below, showing the receptacle (1) of the container that includes an excreted stool (3).



58. O'Neill discloses that "the container is hermetically sealable to reduce the exposure to atmospheric oxygen of a stool deposited therein. The seal may be achieved by means of a lid or cover (e.g. having a snap-fit or screw fitting) for the mouth." *Id.* at page 9, lines 28-33. Figure 3b of O'Neill shows the lid (13) that seals the container of the receptacle (1). Additionally, O'Neill describes that the stool can be collected at home. *Id.* at page 8, lines 7-8

(“the stool is excreted into the container at home and then transported to e.g. a clinic for washing”). In sum, O'Neill discloses the first limitation of claim 1.

3) Second Limitation of Claim 1

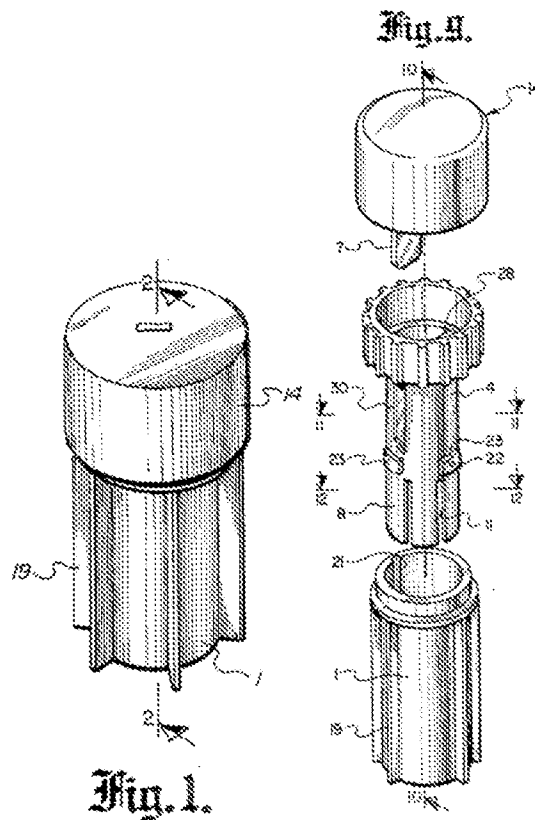
59. The second limitation of claim 1 of the '781 Patent provides “b) removing a portion of the fecal sample to a separate sealable container to produce a removed portion and a remaining portion of the fecal sample ...”.

60. Lapidus '178 and Lapidus '650 each teach removing a portion of the fecal sample to a separate sealable container, which would produce a 'removed portion' and a 'remaining portion' of the fecal sample. Lapidus '178 and Lapidus '650 both teach that “a cross-sectional portion is removed from the voided stool by making one or more sagittal or coronal section through the stool.” Ex. 1002 at column 8, lines 45-49; Ex. 1003 at column 7, lines 17-19. A person of ordinary skill in the art would understand this cross-sectional portion to be a 'removed portion' of the fecal sample recited in claim 1. Lapidus '178 additionally teaches that the 'removed portion' could be in the form of a circumferential surface portion of the voided stool sample or a layer of the voided stool sample that adheres to the surface of a sampling device. Ex. 1002 at column 8, lines 49-52; *id.* at column 9, lines 10-12. In these scenarios, the circumferential surface portion or the layer portion described by Lapidus '178 would correspond to the 'removed portion' of the fecal sample in claim 1. A person of ordinary skill in the art would recognize from the teachings of Lapidus '178 and Lapidus '650 that the portion of the fecal sample left over after removal of a cross-sectional (or a circumferential surface or layer) would be the 'remaining portion' of the fecal sample recited in claim 1.

61. Moreover, Lapidus '178 and Lapidus '650 describe the 'removed portion' of the fecal sample being obtained upon collection of the fecal sample. With reference to Figure 2 (reproduced above), Lapidus '178 and Lapidus '650 each disclose that “[m]eans 8 may be fixedly attached to the support frame 1 or may be removably attached for removal subsequent to deposition of stool. Means 8 may comprise a further means for removing at least a circumferential surface or cross-sectional portion from the voided stool.” Ex. 1002 at column 8, lines 33-37; Ex. 1003 at column 7, lines 61-65. The person of ordinary skill in the art would understand this disclosure to teach that the 'removed portion' of the fecal sample (e.g., a circumferential surface or cross-sectional portion from the voided stool) can be obtained in the conventional toilet upon collection of the voided stool.

62. Additionally, Lapidus '178 and Lapidus '650 both teach that the 'removed portion' of the fecal sample is in a separate sealable container. Lapidus '178 and Lapidus '650 describe that the 'removed portion' (e.g., a cross-sectional sample of stool) is placed in a buffer such as phosphate buffered saline according to "means and materials" that are "generally known in the art" in order to assay the 'removed portion'. Ex. 1002 at column 9, lines 30-34; Ex. 1003 at column 8, lines 5-8. At the time we filed the application that led to Lapidus '650 and Lapidus '178, it was well known in the art to use a separate sealable container to homogenize a fecal sample in appropriate buffers for the purpose of assaying. For instance, U.S. Patent No. 4,859,610 to Maggio (Ex. 1010, "Maggio") is one example of the generally known "means and materials" for homogenization being referred to in Lapidus '178 and Lapidus '650.

63. Maggio describes a device that is "particularly useful for homogenizing and analyzing fecal samples." Ex. 1010 at column 4, lines 7-8. Maggio teaches that the device includes a vessel for placement of a fecal sample and that "[a]fter a measured portion of [fecal sample] is secured by the scoop (8) and introduced into the vessel (1), 2 mls of phosphate buffered saline, pH 7.4 (PBS) is added to the vessel (1)." *Id.* at column 8, lines 39-42. Maggio later describes that "FIG. 1 is a perspective view of a preferred embodiment of the immunoassay incubation device." *Id.* at column 3, lines 33-34. Furthermore, Maggio discloses that "FIG. 9 is an exploded view in perspective of another alternative embodiment of the immunoassay incubation device illustrating a valve for controlling the flow of homogenized sample from the extractor into the incubation chamber." *Id.* at column 3, lines 60-64. Figures 1 and 9 of Maggio, reproduced below, clearly depict a separate sealable container that is configured to contain a fecal sample and a homogenizing buffer. Once again, the foregoing disclosure from Maggio exemplifies the type of "homogenization means and materials" being referred to in Lapidus '178 (Ex. 1002 at column 9, lines 30-34) and Lapidus '650 (Ex. 1003 at column 8, lines 5-8), as the person of ordinary skill in the art would have appreciated from the Lapidus disclosure.



64. Furthermore, any minor differences that may exist between the second limitation of claim 1 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would involve removing a portion of the fecal sample to a separate sealable container such that there would be removed and remaining portions of the sample available for testing. Thus, at a minimum, this aspect of claim 1 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

65. Additionally, De Luca discloses “an extraction and sampling device for collection of faeces samples, particularly for laboratory immunological tests, for qualitative or quantitative determination of one or more analytes, such as haemoglobin...”. Ex. 1006 at [0001]. The devices of De Luca are used for detection of blood proteins such as hemoglobin for use in fecal immunochemical blood tests. Further, De Luca teaches that fecal samples are removed and placed in a separate sealable container to perform the fecal immunochemical blood tests. *Id.* at [0015]. The extraction tube of De Luca includes a container body for containing a buffer

solution and fecal sample, and the container is sealed using a cap, rendering the extraction tube a “separate sealable container.” *Id.* at [0019], [0032].

66. A person of ordinary skill in the art would have been motivated to utilize the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of Lapidus ‘178 or Lapidus ‘650. Specifically, based on the advantages taught in De Luca, the person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer in the extraction tube’s container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test could be performed. Modifying the method of Lapidus ‘178 or of Lapidus ‘650 to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the second limitation of claim 1 based on the combined teachings of Lapidus ‘178 or Lapidus ‘650 in view of De Luca.

67. Ahlquist also teaches removing a portion of the fecal sample to produce a ‘removed portion’ and a ‘remaining portion’ of the fecal sample. The patients in Ahlquist collected stool and then “smeared stool” onto Hemoccult and HemoccultSensa cards. Ex. 1004 at page 4, at ‘Stool Collection and Processing’ heading. This smeared stool sample corresponds to the ‘removed portion’ of the fecal sample in claim 1. The rest of the stool in the plastic bucket disclosed by Ahlquist corresponds to the ‘remaining portion’ of the fecal sample in claim 1. *Id.*

68. A person of ordinary skill in the art would have been motivated to utilize the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of Ahlquist. Ahlquist discloses that “[w]e did not evaluate fecal immunochemical blood tests, and such tests may detect more adenomas than do guaiac tests, because of differences in relative detection rates. *Id.* at page 9, second full paragraph (citations omitted). As discussed above, De Luca teaches the fecal immunochemical blood tests indicated by Ahlquist to provide improved adenoma detection compared to guaiac tests. *See* Ex. 1006 at [0001]. Based on Ahlquist’s suggestion to use fecal immunochemical blood tests to “detect more adenomas,” as well as the advantages taught in De Luca, a person of ordinary skill in the art would have been motivated to

place the removed portion of the fecal sample with a buffer in the extraction tube's container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test could be performed, as taught in DeLuca. Modifying the method of Ahlquist to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the second limitation of claim 1 based on the combined teachings of Ahlquist and De Luca.

69. O'Neill also teaches removing a portion of the fecal sample to produce a 'removed portion' and a 'remaining portion' of the fecal sample. O'Neill discloses that cellular material is removed from the fecal sample via a wash liquid and teaches that this 'removed portion' is placed into sample receptacles for subsequent analysis of DNA, RNA and proteins. In particular, O'Neill discloses that "[w]hen the wash programme ends, the sample rack is pivoted about pivot 47 to bring axis 41 into a vertical position (see Fig. 5). Almost all of wash liquid 44 is then decanted out of receptacle 1 via port 21, foraminous screen 25 substantially preventing larger food residues or other broken off pieces from the stool exiting with the liquid. Coarse sieve 25a removes smaller pieces from the liquid. Finer downstream sieves (not shown) further limit the maximum particle size in the liquid. The liquid is then be divided into standard volumes and received into a plurality of removable (e.g. clip-on) sample receptacles (not shown). The sample receptacles may be a strip of tubes each of e.g. 0.1-0.5 ml volume as can be removed and clipped into a standard microplate format used for clinical analyses. Alternatively the sample receptacles may be individual tubes each of e.g. 1-2 ml volume. The sample rack is then returned to its horizontal position and receptacle 1 is removed and safely disposed of." Ex. 1005 at page 14, lines 18-35. A person of ordinary skill in the art would have understood the individual tubes described by O'Neill to be separate, sealable containers, such as Eppendorf tubes that are used in the art. As such, O'Neill teaches "removing a portion of the fecal sample to a separate sealable container." The rest of the stool in the container corresponds to the 'remaining portion' of the fecal sample recited in claim 1.

70. Furthermore, a person of ordinary skill in the art would have found it obvious to utilize the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of O’Neill. Based on De Luca’s teachings regarding the advantages of such containers, a person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer, as taught in DeLuca. Modifying the method of O’Neill to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the second limitation of claim 1 based on the combined teachings of O’Neill and De Luca.

4) Third Limitation of Claim 1

71. The third limitation of claim 1 of the ‘781 Patent provides “c) combining the removed portion of the fecal sample in the separate sealable container with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample, and sealing the sealable container...”.

72. Lapidus ‘178 and Lapidus ‘650 each teach combining the removed portion of the fecal sample in the separate sealable container with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample, and sealing the sealable container. Lapidus ‘178 and Lapidus ‘650 both describe combining the removed portion of the fecal sample (e.g., the cross-sectional portion) with a buffer. Ex. 1002 at column 9, lines 30-36; Ex. 1003 at column 8, lines 5-21. Lapidus ‘178 and Lapidus ‘650 disclose the buffers as any buffer known to the skilled artisan, such as those that contain detergents and salts, which are well known for preservation of proteins. *Id.* Lapidus ‘178 and Lapidus ‘650 further note that to prevent the denaturation or degradation of blood proteins found in the sample, it would be important to specifically avoid including a proteinase in the buffer that would contain detergents and salts. *Id.*

73. As discussed above, with regard to the second limitation of claim 1, Lapidus ‘178 and Lapidus ‘650 describe that the cross-sectional portion (and, in Lapidus ‘178, circumferential surface portion or layer portion) of the fecal sample can be homogenized in a buffer according to “means and materials” that are generally known in the art. Ex. 1002 at column 9, lines 30-34;

Ex. 1003 at column 8, lines 5-8. These generally known “means and materials” included separate sealable containers for combining a removed portion of a sample with a buffer, as evidenced by Maggio. *See, e.g.*, Maggio, FIGS. 1, 9; column 3, lines 33-34, 60-64; column 4, lines 7-8; column 8, lines 39-42. Accordingly, Lapidus ‘178 and Lapidus ‘650 each taught a person of ordinary skill in the art that the removed portion of the fecal sample and buffer would be combined in a separate sealable container and sealed, according to methods known in the art.

74. Furthermore, any minor differences that may exist between the third limitation of claim 1 and the teachings of Lapidus ‘178 and Lapidus ‘650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus ‘178 and Lapidus ‘650 patents, I envisioned that the methods described in those references could and would involve combining the removed portion of the fecal sample in the separate sealable container with a buffer that prevented denaturation or degradation of blood proteins found in a fecal sample and then sealing the sealable container. Thus, at a minimum, this aspect of claim 1 was obvious in view of the teachings of Lapidus ‘178 and/or Lapidus ‘650.

75. Additionally, as discussed above, De Luca discloses placing fecal in a separate sealable containers, with a buffer solution, to perform the fecal immunochemical blood tests. *See, e.g.*, Ex. 1006 at [0001], [0015], [0019], [0032]. As noted above, a person of ordinary skill in the art would have been motivated to utilize the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of Lapidus ‘178 or Lapidus ‘650. Specifically, based on the advantages taught in De Luca, the person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer in the extraction tube’s container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test could be performed. Modifying the method of Lapidus ‘178 or of Lapidus ‘650 to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the third limitation of claim 1 based on the combined teachings of Lapidus ‘178 or Lapidus ‘650 in view of De Luca.

76. Similarly, a person of ordinary skill in the art would have been motivated to utilize the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of Ahlquist (i.e., the smeared portions). As discussed above, Ahlquist states that fecal immunochemical blood tests provide improved detection of adenomas compared to guaiac tests, while De Luca teaches such fecal immunochemical blood tests. *See* Ex. 1004 at page 9, second full paragraph; Ex. 1006 at [0001]. Based on Ahlquist’s statement suggestion to use fecal immunochemical blood tests to “detect more adenomas,” as well as the advantages taught in De Luca, the person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer in the extraction tube’s container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test could be performed, as taught in DeLuca. Modifying the method of Ahlquist to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the third limitation of claim 1 based on the combined teachings of Ahlquist and De Luca.

77. Likewise, a person of ordinary skill in the art would also have been motivated to utilize the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of O’Neill (i.e., removed cellular material). O’Neill teaches that the removed portion is placed into sample receptacles for subsequent analysis of DNA, RNA and proteins. Ex. 1005 at page 1, lines 15-20. Thus, a person of ordinary skill in the art reading O’Neill would be motivated to stabilize the removed cellular material so that it could provide a more accurate assessment for patients. Specifically, based on the advantages taught in De Luca, the person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer in the extraction tube’s container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test could be performed, as taught in DeLuca. Modifying the method of O’Neill to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of

a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the third limitation of claim 1 based on the combined teachings of O'Neill and De Luca.

5) Fourth Limitation of Claim 1

78. The fourth limitation of claim 1 of the '781 Patent provides "d) combining the remaining portion of the fecal sample in the sealable collection vessel with a stabilizing buffer, and sealing the sealable collection vessel."

79. Lapidus '178 and Lapidus '650 each teach combining the remaining portion of the fecal sample in the sealable collection vessel with a stabilizing buffer, and sealing the sealable collection vessel. As discussed above, with regard to the second limitation of claim 1, a person of ordinary skill in the art would recognize from the teachings of Lapidus '178 and Lapidus '650 that the portion of the fecal sample left over after removal of a cross-sectional (or, in Lapidus '178, a circumferential surface or layer) would be the 'remaining portion' of the fecal sample recited in claim 1. The 'remaining portion' of the fecal sample corresponds to the "stool" in Lapidus '178 and Lapidus '650, which is described as being contained in the receptacle (i.e., the "sealable collection vessel"). Further, Lapidus '178 and Lapidus '650 teach that homogenization buffer can be added to the receptacle containing the stool. Lapidus '178 and Lapidus '650 each state that "[m]ethods of the invention also comprise obtaining a representative (i.e., cross-sectional) *sample of stool* and homogenizing the stool in a buffer, such as a buffer comprising a detergent and a proteinase and optionally a DNase inhibitor. Ex. 1002 at column 3, lines 11-15 (emphasis added); Ex. 1003 at column 2, line 66 – column 3, line 3 (emphasis added). A person of ordinary skill in the art would understand this teaching of Lapidus '178 and Lapidus '650 to refer to two different portions of fecal material – the 'removed portion', or "*sample of stool*", and also the 'remaining portion' of "the stool." Lapidus '178 and Lapidus '650 therefore disclose addition of a buffer to the 'remaining portion', i.e., "the stool."

80. Lapidus '178 and Lapidus '650 teach that the buffer can contain phosphate buffered saline, NaCl or KCl, a detergent such as SDS or triton, and inhibitors of DNA and/or RNA degrading enzymes. Ex. 1002 at column 8, lines 5-16; Ex. 1003 at column 7, lines 31-44. A person of ordinary skill in the art would recognize such a buffer as a "stabilizing buffer."

81. Lapidus '178 and Lapidus '650 also disclose that the receptacle includes sealing means and that it should be sealed "to contain the voided stool sample and any solution added thereto and to prevent the emanation of odors." Ex. 1002 at column 8, lines 17-21; Ex. 1003 at column 8, lines 45-49.

82. Furthermore, any minor differences that may exist between the fourth limitation of claim 1 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would involve combining the remaining portion of the fecal sample in the sealable collection vessel with a stabilizing buffer and then sealing the sealable collection vessel. Thus, at a minimum, this aspect of claim 1 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

83. Additionally, Olson discloses that addition of a stabilizing buffer (Exact buffer A) to stool samples provided a significant increase in recovery of DNA compared with samples incubated without buffer. Olson describes development of "a multitarget, fecal DNA screening assay that detects the presence of gene-specific mutations and long DNA fragments associated with colorectal cancer (CRC)." Ex. 1007 at Abstract. Olson teaches that "[t]he 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 t0 the average recovery was 14,891 copies/10 mL, 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 t0. 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 t0 samples (Table 3)." *Id.* at page 186, first column, second full paragraph. In conclusion, Olson states that "DNA can be stabilized by addition of buffer to the sample, leading to increased assay sensitivity. *Id.* at Abstract.

84. A person of ordinary skill in the art would have been motivated to combine the stabilizing buffer of Olson with the 'remaining portion' of the fecal sample of Lapidus '178 or Lapidus '650. Specifically, based on the advantages taught in Olson, the person of ordinary skill

in the art would have been motivated to use the stabilizing buffer taught in Olson to improve DNA assays on the 'remaining portion' of the fecal sample. Modifying the method of Lapidus '178 or of Lapidus '650 to include these teachings of Olson would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the fourth limitation of claim 1 based on the combined teachings of Lapidus '178 or Lapidus '650 in view of Olson.

85. Furthermore, Ahlquist teaches that, after a portion of the fecal sample is removed (by smearing onto the guaiac-based Hemocult and HemocultSensa cards), the remaining portion of the fecal sample is "sealed in a bucket" and express-shipped to the testing facility, along with the Hemocult and HemocultSensa cards. Ex. 1004 at page 4, at 'Stool Collection and Processing' heading. Although Ahlquist does not teach addition of a stabilizing buffer to the remaining portion of the fecal sample, Ahlquist states that "[t]echnical advances could improve DNA assay sensitivity" on the stools and that "[m]ethods to increase stool DNA capture are needed, and some have been reported." *Id.* at page 9, third full paragraph (citations omitted). To this end, Ahlquist states that its authors and other researchers "have shown that adding a preservative to stool at the time of defecation can prevent degradation of long DNA." *Id.* Thus, a person of ordinary skill in the art reading Ahlquist would have been motivated to add a preservative as a stabilizing buffer to the stool at the time of defecation in order to prevent degradation of nucleic acids. As discussed above, Olson teaches such a stabilizing buffer (Exact buffer A). Ex. 1007 at Abstract; page 186, first column, second full paragraph.

86. A person of ordinary skill in the art would have been motivated to combine the stabilizing buffer of Olson with the 'remaining portion' of the fecal sample of Ahlquist, prior to sealing them in the bucket. Specifically, based on the express suggestion in Ahlquist, as well as the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to use the stabilizing buffer taught in Olson to improve DNA assays on the 'remaining portion' of the fecal sample in Ahlquist. Modifying the method of Ahlquist to include these teachings of Olson would have required only minor changes to general methods to stabilize fecal samples

with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the fourth limitation of claim 1 based on the combined teachings of Ahlquist and Olson.

87. Additionally, O'Neill teaches that, after a portion of the fecal sample is removed (by washing to remove cellular material), the remaining portion of the fecal sample is stays in the sealed container. O'Neill states that a typical total stool has a mass of 100-500 grams and that the removed cellular material has a mass of about 50-1000 mg. Ex. 1005 at page 15, lines 1-4. Thus, a person of ordinary skill in the art would understand that O'Neill teaches a remaining portion of the stool of at least 99 grams that remains in the container. Although O'Neill does not teach addition of a stabilizing buffer to the remaining portion of the fecal sample, a person of ordinary skill in the art would have been motivated to stabilize the remaining stool sample so that subsequent analyses could be made and/or improved. As discussed above, Olson teaches such a stabilizing buffer (Exact buffer A). Ex. 1007 at Abstract; page 186, first column, second full paragraph.

88. A person of ordinary skill in the art would have been motivated to combine the stabilizing buffer of Olson with the 'remaining portion' of the fecal sample of O'Neill. Specifically, based on the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to use the stabilizing buffer taught in Olson to improve DNA assays on the 'remaining portion' of the fecal sample in O'Neill. Modifying the method of O'Neill to include these teachings of Olson would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the fourth limitation of claim 1 based on the combined teachings of O'Neill and Olson.

6) Conclusions Regarding Claim 1

89. For the reasons discussed above, it is my opinion that Lapidus '178 described each and every element of claim 1 arranged in the same way as in that claim. Therefore, Lapidus '178 anticipated claim 1.

90. For the reasons discussed above, it is also my opinion that Lapidus '178 suggested each and every element of claim 1 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '178 rendered claim 1 obvious.

91. For the reasons discussed above, it is also my opinion that Lapidus '650 described each and every element of claim 1 arranged in the same way as in that claim. Therefore, Lapidus '650 anticipated claim 1.

92. For the reasons discussed above, it is also my opinion that Lapidus '650 suggested each and every element of claim 1 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '650 rendered claim 1 obvious.

93. For the reasons discussed above, it is also my opinion that Lapidus '178, De Luca, and Olson collectively taught or suggested each and every element of claim 1 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 1. Therefore, Lapidus '178, De Luca, and Olson rendered claim 1 obvious.

94. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, and Olson collectively taught or suggested each and every element of claim 1 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 1. Therefore, Lapidus '650, De Luca, and Olson rendered claim 1 obvious.

95. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, and Olson collectively taught or suggested each and every element of claim 1 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 1. Therefore, Ahlquist, De Luca, and Olson rendered claim 1 obvious.

96. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 1 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those

teachings to arrive at the subject matter of claim 1. Therefore, O'Neill, De Luca, and Olson rendered claim 1 obvious.

B. Claim 2 Was Anticipated and Obvious

97. Claim 2 of the '781 Patent provides "[t]he method of claim 1, further comprising delivering the sealable container containing the removed portion of the fecal sample and said buffer and the sealable collection vessel containing the remaining portion of the fecal sample and said stabilizing buffer to a medical diagnostics laboratory."

98. Lapidus '178 and Lapidus '650 each teach "that "[s]tool is voided into a receptacle that is preferably small enough to be transported to a testing facility." Ex. 1002 at column 7, lines 63-64; Ex. 1003 at column 7, lines 24-26. A person of ordinary skill in the art would understand that 'a testing facility' as described in Lapidus '178 and Lapidus '650 would correspond to 'a medical diagnostics laboratory' according to the usual meaning in the field. Thus, both Lapidus '178 and Lapidus '650 disclose claim 2.

99. Furthermore, any minor differences that may exist between claim 2 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would involve delivering the sealable container containing the removed portion of the fecal sample and said buffer and the sealable collection vessel containing the remaining portion of the fecal sample and said stabilizing buffer to a medical diagnostics laboratory. Thus, at a minimum, claim 2 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

100. Furthermore, Ahlquist discloses "[p]atients collected 3 stools by using plastic buckets mounted to the toilet seat. Promptly after each individual collection, patients smeared stool onto both windows of their Hemoccult and HemoccultSensa cards and then express-shipped smeared cards and the whole stool (sealed in a bucket in an insulated container cooled with ice packs) to the Mayo Clinic in Rochester, Minnesota. Ex. 1004 at page 4, at 'Stool Collection and Processing' heading. A person of ordinary skill in the art would understand that the Mayo Clinic as taught by Ahlquist would indicate 'a medical diagnostics laboratory' according to the usual meaning in the field. Thus, Ahlquist teaches claim 2.

101. Moreover, O'Neill teaches that "[i]f the stool is excreted into the container at home and then transported to e.g. a clinic for washing, the container has the further advantage of holding the stool reasonably securely in the channel so that transportation damage to the stool surface is reduced or avoided." *Id.* at page 8, lines 7-11. A person of ordinary skill in the art would understand that the clinic as taught by O'Neill would indicate 'a medical diagnostics laboratory' according to the usual meaning in the field. Thus, O'Neill teaches claim 2.

102. As taught by Lapidus '178, Lapidus '650, Ahlquist, and O'Neill, it was routine in the art for the method of processing to include collection of fecal samples at a place other than a medical diagnostics laboratory and then delivery of the fecal samples to the medical diagnostics laboratory. For instance, a person of ordinary skill in the art would be motivated to utilize the teachings of Lapidus '178, Lapidus '650, Ahlquist, and/or O'Neill to collect fecal samples at one location and then deliver them to a medical diagnostics laboratory since each reference suggests that these steps could be undertaken. All such teachings were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results.

103. In summary, for the reasons discussed above, it is my opinion that Lapidus '178 described each and every element of claim 2. Therefore, Lapidus '178 anticipated claim 2.

104. For the reasons discussed above, it is also my opinion that Lapidus '178 suggested each and every element of claim 2 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '178 rendered claim 2 obvious.

105. For the reasons discussed above, it is also my opinion that Lapidus '650 described each and every element of claim 2. Therefore, Lapidus '650 anticipated claim 2.

106. For the reasons discussed above, it is also my opinion that Lapidus '650 suggested each and every element of claim 2 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '650 rendered claim 2 obvious.

107. For the reasons discussed above, it is also my opinion that Lapidus '178, De Luca, and Olson collectively taught or suggested each and every element of claim 2 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 2. Therefore, Lapidus '178, De Luca, and Olson rendered claim 2 obvious.

108. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, and Olson collectively taught or suggested each and every element of claim 2 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 2. Therefore, Lapidus '650, De Luca, and Olson rendered claim 2 obvious.

109. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, and Olson collectively taught or suggested each and every element of claim 2 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 2. Therefore, Ahlquist, De Luca, and Olson rendered claim 2 obvious.

110. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 2 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 2. Therefore, O'Neill, De Luca, and Olson rendered claim 2 obvious.

C. Claim 3 Was Anticipated and Obvious

1) Preamble of Claim 3

111. The preamble of claim 3 of the '781 Patent provides "[a] method of processing a fecal sample, the method comprising ...". As discussed above in relation to claim 1, a person of ordinary skill in the art would have understood that each of Lapidus '178, Lapidus '650, Ahlquist, and O'Neill teach methods of processing a fecal sample. As such, each of Lapidus '178, Lapidus '650, Ahlquist, and O'Neill teach the preamble of claim 3.

2) First Limitation of Claim 3

112. The first limitation of claim 3 of the '781 Patent provides "a) obtaining a pair of portions of a fecal sample collected from a human subject, the pair of portions comprising: i) a sealed sealable container containing a removed portion of a fecal sample and a buffer; and ii) a sealed sealable collection vessel containing a remaining portion of a fecal sample and a stabilizing buffer, the pair of portions obtained by the method of claim 1 ...". As discussed above in relation to claim 1, a person of ordinary skill in the art would have understood that each of Lapidus '178, Lapidus '650, Ahlquist, and O'Neill taught and/or suggested obtaining a pair of

portions of a fecal sample collected from a human subject that are obtained by the method of claim 1.

113. Accordingly, both Lapidus '178 and Lapidus '650 disclose the first limitation of claim 3. Furthermore, any minor differences that may exist between the first limitation of claim 3 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Moreover, Ahlquist discloses the first limitation of claim 3. In addition, O'Neill discloses the first limitation of claim 3.

3) Second Limitation of Claim 3

114. The second limitation of claim 3 of the '781 Patent provides "b) testing the removed portion of the fecal sample for an amount of a blood protein present in the removed portion ...".

115. Lapidus '178 and Lapidus '650 each testing the removed portion of the fecal sample for an amount of a blood protein present in the removed portion. Lapidus '178 and Lapidus '650 both teach that after sample (e.g., a cross-sectional sample) "of stool is obtained, it may be homogenized by known methods to distribute cells and cellular debris throughout the sample. An assay then is performed on the homogenate, or an extract of the homogenate, to detect the presence of cells and/or cellular debris in the sample. The assay may be any one or a combination of histological cellular assays, antibody based immunoassays (or other formats) designed to detect the presence of a molecule characteristic of transformation such as a protein, or DNA-based assays for detecting mutations or genetic characteristics indicative of colorectal cancer." Ex. 1002 at column 6, lines 45-56; Ex. 1003 at column 6, lines 1-11. Lapidus '178 and Lapidus '650 additionally teach that "[i]f a fecal occult blood assay is to be performed, the buffer may contain chemical compounds which react with blood to produce a color, the intensity of which can be measured. Buffers useful for detecting the presence of fecal occult blood are known in the art." Ex. 1002 at column 9, lines 40-45; Ex. 1003 at column 8, lines 13-18.

116. Furthermore, any minor differences that may exist between the second limitation of claim 3 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would involve testing the removed portion of the fecal sample for an

amount of a blood protein present in the removed portion. Thus, at a minimum, this aspect of claim 3 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

117. In addition, De Luca discloses “collection of faeces samples, particularly for laboratory immunological tests, for qualitative or quantitative determination of one or more analytes, such as haemoglobin...”. Ex. 1006 at [0001]. De Luca discloses “[t]he extraction tube for collection of faeces samples, according to the invention, comprises a container body hollow on the inside for containing a buffer solution and faeces samples, a top cap applied to the top end of the container body and provided with a threaded small rod for sampling of faeces samples and a bottom cap applied removably to the bottom end of the container body. *Id.* at [0019]. Further, De Luca discloses that “the extraction tube [] is positioned in a special housing [] of an automatic machine for immunological tests and then the bottom cap [] is removed so as to allow removal of the buffer solution [] containing the faeces samples by a probe of the automatic machine.” *Id.* at [0048].

118. A person of ordinary skill in the art would have been motivated to utilize the laboratory immunological tests of De Luca to analyze hemoglobin in the fecal sample of Lapidus '178 and/or Lapidus '650. Lapidus '178 and Lapidus '650 disclose that testing blood proteins was a goal of their analyses. As discussed above, De Luca teaches fecal immunochemical blood tests that can improve improved adenoma detection. *See* Ex. 1006 at [0001]. These advantages taught in De Luca would have motivated a person of ordinary skill in the art to place the removed portion of the fecal sample with a buffer in the extraction tube's container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test could be performed, as taught in DeLuca. Modifying the method of Lapidus '178 or Lapidus '650 to include these teachings of De Luca would have required only minor changes to general methods to test fecal samples for hemoglobin, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the second limitation of claim 3 based on the combined teachings of Lapidus '178 or Lapidus '650 and De Luca.

119. Ahlquist teaches that “[p]atients collected 3 stools by using plastic buckets mounted to the toilet seat. Promptly after each individual collection, patients smeared stool onto

both windows of their Hemocult and HemocultSensa cards and then express-shipped smeared cards and the whole stool (sealed in a bucket in an insulated container cooled with ice packs) to the Mayo Clinic in Rochester, Minnesota. Ex. 1004 at page 4, at 'Stool Collection and Processing' heading.

120. A person of ordinary skill in the art would have been motivated to analyze hemoglobin in the fecal sample of Ahlquist. Ahlquist discloses that "[w]e did not evaluate fecal immunochemical blood tests, and such tests may detect more adenomas than do guaiac tests, because of differences in relative detection rates. *Id.* at page 9, second full paragraph (citations omitted). As discussed above, De Luca teaches the fecal immunochemical blood tests indicated by Ahlquist to provide improved adenoma detection compared to guaiac tests by testing for a blood protein such as hemoglobin. *See* Ex. 1006 at [0001]. Based on Ahlquist's suggestion to use fecal immunochemical blood tests to "detect more adenomas," as well as the advantages taught in De Luca, a person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer in the extraction tube's container body to prevent denaturation or degradation of blood proteins so that a fecal immunochemical blood test on blood proteins could be performed, as taught in DeLuca. Modifying the method of Ahlquist to include these teachings of De Luca would have required only minor changes to general methods to stabilize fecal samples with buffer in sealable containers, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the second limitation of claim 3 based on the combined teachings of Ahlquist and De Luca.

121. O'Neill teaches "a method and apparatus for removing cellular material from faecal stools and a container for collecting faecal stools." Ex. 1005 at page 1, lines 5-7. O'Neill discloses that "[e]xfoliated epithelial cells consist of cellular components, such as DNA, RNA and proteins, which potentially provide a multiplicity of approaches to identifying and monitoring unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis. The facile recovery of such cells makes possible the application of a wide variety of test procedures for qualitative and/or quantitative analysis of, for example, DNA alterations, gene expression and enzyme activity." *Id.* at page 1, lines 15-24.

122. Furthermore, a person of ordinary skill in the art would have found it obvious to test for a blood protein such as hemoglobin via the separate sealable container of De Luca to receive the ‘removed portion’ of the fecal sample of O’Neill. Based on De Luca’s teachings regarding the advantages of such containers, a person of ordinary skill in the art would have been motivated to place the removed portion of the fecal sample with a buffer, as taught in DeLuca, and test for a blood protein such as hemoglobin. Modifying the method of O’Neill to include these teachings of De Luca would have required only minor changes to general methods to test for a blood protein such as hemoglobin, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the second limitation of claim 3 based on the combined teachings of O’Neill and De Luca.

4) Third Limitation of Claim 3

123. The third limitation of claim 3 of the ‘781 Patent provides “c) extracting nucleic acid from the remaining portion of the fecal sample ...”.

124. Lapidus ‘178 and Lapidus ‘650 each teach extracting nucleic acid from the remaining portion of the fecal sample. Lapidus ‘178 and Lapidus ‘650 both teach diagnostic assays can be performed on a whole stool or a portion that “contains sufficient material to allow subsequent diagnostic assays to be performed.” Ex. 1002 at column 9, lines 26-29; Ex. 1003 at column 9, lines 21-24. Lapidus ‘178 and Lapidus ‘650 teach that the an illustrative “assay may be any one or a combination of histological cellular assays, antibody based immunoassays (or other formats) designed to detect the presence of a molecule characteristic of transformation such as a protein, or DNA-based assays for detecting mutations or genetic characteristics indicative of colorectal cancer. Known assay protocols, those disclosed herein or in U.S. Pat. No. 5,670,325 or assays hereafter developed may be used in the practice of the invention. Non-limiting examples of useful known assay protocols include those disclosed in U.S. Pat. Nos. 5,137,806 (detection of sequences in selected DNA molecules), 5,348,855 (assay for nucleic acid sequences), 5,512,441 (detection of mutant alleles), 5,272,057 and 5,380,645 (RFLP analysis), 5,527,676 (detection of p53 gene sequences), 5,330,892 (detection of MCC gene sequences), 5,352,775 (detection of APC gene sequences), 5,532,108 (detection of DCC gene sequences), and in WO96/08514

(monoclonal antibodies against human colon carcinoma-associated antigens), the disclosures of each of which are incorporated by reference herein.” Ex. 1002 at column 6, line 50 – column 7, line 2; Ex. 1003 at column 6, lines 6-28. Lapidus ‘178 and Lapidus ‘650 each describe “[m]ethods of the invention are used to prepare a stool sample followed by detection of a deletion or other mutation in the p53 tumor suppressor gene. ... DNA or RNA may optionally be isolated from the sample according to methods known in the art.” Ex. 1002 at column 9, line 62 – column 10, line 6; Ex. 1003 at column 8, lines 39-50.

125. As such, Lapidus ‘178 and Lapidus ‘650 each describe that nucleic acid (e.g., DNA or RNA) can be extracted from fecal samples and subjected to “any one or a combination of histological cellular assays, antibody based immunoassays (or other formats) designed to detect the presence of a molecule characteristic of transformation such as a protein, or DNA-based assays for detecting mutations or genetic characteristics indicative of colorectal cancer” including the assays described in any of the at least 11 prior art references incorporated by reference into Lapidus ‘178 and Lapidus ‘650. Accordingly, Lapidus ‘178 and Lapidus ‘650 each taught a person of ordinary skill in the art a number of available assays capable of extracting nucleic acid from the remaining portion of the fecal sample, according to methods known in the art.

126. Furthermore, any minor differences that may exist between the third limitation of claim 3 and the teachings of Lapidus ‘178 and Lapidus ‘650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus ‘178 and Lapidus ‘650 patents, I envisioned that the methods described in those references could and would involve extracting nucleic acid from the remaining portion of the fecal sample. Thus, at a minimum, this aspect of claim 3 was obvious in view of the teachings of Lapidus ‘178 and/or Lapidus ‘650.

127. Additionally, as discussed above, Olson discloses development of “a multitarget, fecal DNA screening assay that detects the presence of gene-specific mutations and long DNA fragments associated with colorectal cancer (CRC).” Ex. 1007 at Abstract. Olson discloses that “[t]he 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 t0 the average recovery was 14,891 copies/10 mL, 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 t0. 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 t0 samples (Table 3).” *Id.* at page 186, first column, second full paragraph. In conclusion, Olson states that “DNA can be stabilized by addition of buffer to the sample, leading to increased assay sensitivity. *Id.* at Abstract.

128. A person of ordinary skill in the art would have been motivated to utilize the methods of Olson to extract nucleic acid from fecal samples. Specifically, based on the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to place the remaining portion of the fecal sample in a buffer to stabilize the nucleic acids in order to provide for increased assay sensitivity. Modifying the method of Lapidus ‘178 or of Lapidus ‘650 to include these teachings of Olson would have required only minor changes to general methods to stabilize fecal samples with buffer, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the third limitation of claim 3 based on the combined teachings of Lapidus ‘178 or Lapidus ‘650 in view of Olson.

129. Similarly, a person of ordinary skill in the art would have been motivated to utilize the buffers of Olson to stabilize the DNA in the ‘remaining portion’ of the fecal sample of Ahlquist. As discussed above, states that “[t]echnical advances could improve DNA assay sensitivity” on the stools and that “[m]ethods to increase stool DNA capture are needed, and some have been reported.” Ex. 1004 at page 9, third full paragraph (citations omitted). Ahlquist states that its authors and other researchers “have shown that adding a preservative to stool at the time of defecation can prevent degradation of long DNA.” *Id.* Based on Ahlquist’s suggestion to improve DNA assay sensitivity, as well as the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to place the remaining portion of the fecal sample in a buffer to lead to increased DNA assay sensitivity, as taught in Olson. Modifying the method of Ahlquist to include these teachings of Olson would have required only minor changes to general methods to stabilize fecal samples with buffer, all of which were well within the

knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the third limitation of claim 3 based on the combined teachings of Ahlquist and Olson.

130. Likewise, a person of ordinary skill in the art would also have been motivated to utilize the buffers of Olson to stabilize the DNA in the ‘remaining portion’ of the fecal sample of O’Neill (i.e., removed cellular material). O’Neill teaches that the removed portion is placed into sample receptacles for subsequent analysis of DNA, RNA and proteins. Ex. 1005 at page 1, lines 15-20. Thus, a person of ordinary skill in the art reading O’Neill would be motivated to stabilize the removed cellular material so that it could provide a more accurate DNA assay and subsequent assessment for patients. Specifically, based on the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to place the remaining portion of the fecal sample in a buffer to provide increased DNA assay sensitivity, as taught in Olson. Modifying the method of O’Neill to include these teachings of Olson would have required only minor changes to general methods to stabilize fecal samples with buffer, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the third limitation of claim 3 based on the combined teachings of O’Neill and Olson.

5) Fourth Limitation of Claim 3

131. The fourth limitation of claim 3 of the ‘781 Patent provides “d) testing the nucleic acid for an amount of a human nucleic acid.”

132. Lapidus ‘178 and Lapidus ‘650 each teach a protocol entitled “1. Method for Quantitation of Target and Reference Polynucleotides.” Ex. 1002 at column 11, lines 19-20; Ex. 1003 at column 9, lines 63-65. Here, Lapidus ‘178 and Lapidus ‘650 describe “[e]nhanced quantification of binding events between hybridization probes and target or reference is accomplished by coupling hybridization probes to particles, such as beads (hybridization beads). In order to obtain a precise quantitative measure of the amount of a polynucleotide in a sample, hybridization beads are constructed such that each bead has attached thereto a single

oligonucleotide probe.” Ex. 1002 at column 7, lines 21-27; Ex. 1003 at column 9, lines 66 – column 10, line 5. A person of ordinary skill in the art would recognize a ‘polynucleotide’ is a synonym for a ‘nucleic acid.’ Thus, Lapidus ‘178 and Lapidus ‘650 each teach that human nucleic acid can be quantitated according to its described assays.

133. Furthermore, any minor differences that may exist between the fourth limitation of claim 3 and the teachings of Lapidus ‘178 and Lapidus ‘650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus ‘178 and Lapidus ‘650 patents, I envisioned that the methods described in those references could and would involve testing the nucleic acid for an amount of a human nucleic acid. Thus, at a minimum, this aspect of claim 3 was obvious in view of the teachings of Lapidus ‘178 and/or Lapidus ‘650.

134. Additionally, Olson development of “a multitarget, fecal DNA screening assay that detects the presence of gene-specific mutations and long DNA fragments associated with colorectal cancer (CRC).” Ex. 1007 at Abstract. Olson discloses that “[t]he 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 t0 the average recovery was 14,891 copies/10 mL, 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 t0. 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 t0 samples (Table 3).” *Id.* at page 186, first column, second full paragraph. In conclusion, Olson states that “DNA can be stabilized by addition of buffer to the sample, leading to increased assay sensitivity. *Id.* at Abstract.

135. A person of ordinary skill in the art would have been motivated to combine the multitarget, fecal DNA screening assay of Olson to test nucleic acid in fecal samples of Lapidus ‘178 or Lapidus ‘650 for an amount of a human nucleic acid. Specifically, based on the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to use the stabilizing buffer taught in Olson to improve the multitarget, fecal DNA screening assays in order to test the presence of gene-specific mutations and long DNA fragments associated with

colorectal cancer. Modifying the method of Lapidus '178 or of Lapidus '650 to include these teachings of Olson would have required only minor changes to general methods to test nucleic acid for an amount of a human nucleic acid, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the fourth limitation of claim 3 based on the combined teachings of Lapidus '178 or Lapidus '650 in view of Olson.

136. Furthermore, as discussed above, Ahlquist teaches testing the nucleic acid for an amount of a human nucleic acid. Ahlquist discloses that “[a]ll assays were polymerase chain reaction-based and were run at EXACT Sciences. Stool DNA test 1 was performed as described in Imperiale and colleagues’ study (21). The marker panel for SDT-1 included 21 tumor-specific point mutations (3 on the Kras gene, 10 on the APC gene, and 8 on the p53 gene); the microsatellite-instability marker BAT-26; and long DNA, a marker for delayed apoptosis, which is characteristic of exfoliated neoplastic colonocytes (12). For SDT-2, sequence-specific DNA markers were detected by acrylamide gel electrophoresis, as described by Whitney and colleagues (24); the panel consisted of 3 tumor-specific markers broadly informative for both colorectal cancer and adenomas (25): K-ras mutations, scanning of APC mutator cluster regions, and methylation of the vimentin gene. We used methods described elsewhere to detect mutant Kras (12), APC scanning (25), and vimentin gene methylation (20) assays. We defined any positive component marker result according to the manufacturer’s preestablished criteria as a positive test result. Ex. 1004, pages 4-5, at ‘DNA testing’ heading. A person of ordinary skill in the art reading Ahlquist would understand that Ahlquist teaches testing nucleic acid for an amount of a human nucleic acid because all of these genes would entail human nucleic acid.

137. O’Neill discloses “a method and apparatus for removing cellular material from faecal stools and a container for collecting faecal stools.” Ex. 1005 at page 1, lines 5-7. O’Neill discloses that “[e]xfoliated epithelial cells consist of cellular components, such as DNA, RNA and proteins, which potentially provide a multiplicity of approaches to identifying and monitoring unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis. The facile recovery of such cells makes possible the application of a wide variety of test procedures for qualitative and/or quantitative analysis of, for example, DNA

alterations, gene expression and enzyme activity.” *Id.* at page 1, lines 15-24. Further, O’Neill discloses that “[t]he wash solution was as previously described by A. Loktionov et al. (referenced above), but the stool wash was passed through, a 250 μ m instead of a 125 μ m sieve. Cell clean-up was performed robotically on a TECAN™ Genesis RSP 150 and then DNA was released by mechanical cell disruption in the presence of 0.9%SDS as lysing agent. Following dilution to prevent quenching, fluorimetric DNA assay was performed with a TECAN™ Spectrafluor Plus using PicoGreen (Molecular Probes™) to yield the stool DNA index (SDNAI, defined as nanograms of DNA per ml of stool wash). The effects of pre-soaking, rocking angle, mechanical and bacterial damage, stool age, and wash volume were studied, and the results are given below.” *Id.* at page 16, lines 7-19.

138. A person of ordinary skill in the art would have been motivated to combine the stabilizing buffer of Olson with the removed cellular material of the fecal sample of O’Neill. Specifically, based on the advantages taught in Olson, the person of ordinary skill in the art would have been motivated to use the stabilizing buffer taught in Olson to improve DNA assays, which include testing nucleic acid for an amount of human nucleic acid. Modifying the method of O’Neill to include these teachings of Olson would have required only minor changes to general methods of human nucleic acid detection, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to implement the fourth limitation of claim 3 based on the combined teachings of O’Neill and Olson.

6) Conclusions Regarding Claim 3

139. For the reasons discussed above, it is my opinion that Lapidus ‘178 described each and every element of claim 3 arranged in the same way as in that claim. Therefore, Lapidus ‘178 anticipated claim 3.

140. For the reasons discussed above, it is also my opinion that Lapidus ‘178 suggested each and every element of claim 3 to those skilled in the art prior to the ‘781 Patent’s filing. Therefore, Lapidus ‘178 rendered claim 3 obvious.

141. For the reasons discussed above, it is also my opinion that Lapidus '650 described each and every element of claim 3 arranged in the same way as in that claim. Therefore, Lapidus '650 anticipated claim 3.

142. For the reasons discussed above, it is also my opinion that Lapidus '650 suggested each and every element of claim 3 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '650 rendered claim 3 obvious.

143. For the reasons discussed above, it is also my opinion that Lapidus '178, De Luca, and Olson collectively taught or suggested each and every element of claim 3 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 3. Therefore, Lapidus '178, De Luca, and Olson rendered claim 3 obvious.

144. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, and Olson collectively taught or suggested each and every element of claim 3 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 3. Therefore, Lapidus '650, De Luca, and Olson rendered claim 3 obvious.

145. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, and Olson collectively taught or suggested each and every element of claim 3 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 3. Therefore, Ahlquist, De Luca, and Olson rendered claim 3 obvious.

146. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 3 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 3. Therefore, O'Neill, De Luca, and Olson rendered claim 3 obvious.

D. Claim 4 Was Anticipated and Obvious

147. Claim 4 of the '781 Patent provides "[t]he method of claim 3, wherein testing the nucleic acid comprises determining expression from a human gene."

148. Lapidus '178 and Lapidus '650 each teach “methods of the invention comprise detection of characteristics of such transformed cells, including, for example, mutations, proteins expressed uniquely or in altered amounts in transformed cells, and blood.” Ex. 1002 at column 2, lines 59-63; Ex. 1003 at column 2, lines 50-54. Further, Lapidus '178 and Lapidus '650 teach “DNA or RNA may be isolated from the homogenate using methods known in the art. Subsequent tests may be performed on the isolated DNA and RNA.” Ex. 1002 at column 9, lines 47-49; Ex. 1003 at column 8, lines 22-25. This indicates that nucleic acid (e.g., DNA or RNA) was tested to determine expression from a human gene. In sum, both Lapidus '178 and Lapidus '650 disclose claim 4.

149. Furthermore, any minor differences that may exist between claim 4 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would involve testing the nucleic acid by determining expression from a human gene. Thus, at a minimum, claim 4 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

150. Furthermore, Olson teaches development of “a multitarget, fecal DNA screening assay that detects the presence of gene-specific mutations and long DNA fragments associated with colorectal cancer (CRC).” Ex. 1007 at Abstract.

151. In addition, Ahlquist discloses that “[a]ll assays were polymerase chain reaction–based and were run at EXACT Sciences. Stool DNA test 1 was performed as described in Imperiale and colleagues’ study (21). The marker panel for SDT-1 included 21 tumor-specific point mutations (3 on the Kras gene, 10 on the APC gene, and 8 on the p53 gene); the microsatellite-instability marker BAT-26; and long DNA, a marker for delayed apoptosis, which is characteristic of exfoliated neoplastic colonocytes (12). For SDT-2, sequence-specific DNA markers were detected by acrylamide gel electrophoresis, as described by Whitney and colleagues (24); the panel consisted of 3 tumor-specific markers broadly informative for both colorectal cancer and adenomas (25): K-ras mutations, scanning of APC mutator cluster regions, and methylation of the vimentin gene. We used methods described elsewhere to detect mutant Kras (12), APC scanning (25), and vimentin gene methylation (20) assays. We defined any positive component marker result according to the manufacturer’s preestablished criteria as a positive test result. Ex. 1004, pages 4-5, at ‘DNA testing’ heading. Further, Ahlquist discloses

“we examined SDT-2 (which targets a potentially more informative marker panel) in a more limited manner. Stool DNA test 2 had significantly better neoplasm detection rates than did the fecal blood tests or SDT-1. We accounted for the higher detection rates of SDT-2 by better detection of advanced adenomas, a finding of particular importance for cancer prevention.” *Id.* at page 9, first full paragraph.

152. Moreover, O’Neill discloses “a method and apparatus for removing cellular material from faecal stools and a container for collecting faecal stools.” Ex. 1005 at page 1, lines 5-7. O’Neill discloses that “[e]xfoliated epithelial cells consist of cellular components, such as DNA, RNA and proteins, which potentially provide a multiplicity of approaches to identifying and monitoring unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis. The facile recovery of such cells makes possible the application of a wide variety of test procedures for qualitative and/or quantitative analysis of, for example, DNA alterations, gene expression and enzyme activity.” *Id.* at page 1, lines 15-24.

153. As taught by Olson, Ahlquist, and/or O’Neill, it was routine in the art to test nucleic acid (e.g., DNA or RNA) to determine expression from a human gene. For instance, a person of ordinary skill in the art would be motivated to utilize the teachings of Olson to develop a DNA screening assay from fecal samples, starting from any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill because Olson is in the same field as these references. It would be routine to adapt the nucleic acid testing methods of any of any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with the teachings of Olson in order to investigate nucleic acid such as DNA or RNA in order to detect “the presence of gene-specific mutations and long DNA fragments associated with colorectal cancer” as suggested by Olson. Ex. 1007 at Abstract. Modifying the method of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill to include these teachings of Olson would have required only minor changes to general methods, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 4 based on the combined teachings of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with De Luca and Olson.

154. In summary, for the reasons discussed above, it is my opinion that Lapidus ‘178 described each and every element of claim 4. Therefore, Lapidus ‘178 anticipated claim 4.

155. For the reasons discussed above, it is also my opinion that Lapidus '178 suggested each and every element of claim 4 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '178 rendered claim 4 obvious.

156. For the reasons discussed above, it is also my opinion that Lapidus '650 described each and every element of claim 4. Therefore, Lapidus '650 anticipated claim 4.

157. For the reasons discussed above, it is also my opinion that Lapidus '650 suggested each and every element of claim 4 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '650 rendered claim 4 obvious.

158. For the reasons discussed above, it is also my opinion that Lapidus '178, De Luca, and Olson collectively taught or suggested each and every element of claim 4 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 4. Therefore, Lapidus '178, De Luca, and Olson rendered claim 4 obvious.

159. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, and Olson collectively taught or suggested each and every element of claim 4 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 4. Therefore, Lapidus '650, De Luca, and Olson rendered claim 4 obvious.

160. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, and Olson collectively taught or suggested each and every element of claim 4 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 4. Therefore, Ahlquist, De Luca, and Olson rendered claim 4 obvious.

161. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 4 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 4. Therefore, O'Neill, De Luca, and Olson rendered claim 4 obvious.

E. Claim 5 Was Obvious

162. Claim 5 of the '781 Patent provides "[t]he method of claim 4, wherein determining expression from the human gene comprises testing the nucleic acid for the presence of human DNA having an epigenetic modification." As stated above, a person of ordinary skill in the art would understand the '781 Patent's definition of "epigenetic modification" as the plain and ordinary meaning of the term (i.e., "any alteration in the DNA, generally resulting in diminished gene expression, which is mediated by mechanisms other than alterations in the primary nucleotide sequence of a gene.").

163. Ahlquist discloses that, for evaluation of the second stool DNA test (SDT-1), "sequence-specific DNA markers were detected by acrylamide gel electrophoresis, as described by Whitney and colleagues (24); the panel consisted of 3 tumor-specific markers broadly informative for both colorectal cancer and adenomas (25): K-ras mutations, scanning of APC mutator cluster regions, and methylation of the vimentin gene. We used methods described elsewhere to detect mutant Kras (12), APC scanning (25), and vimentin gene methylation (20) assays." Ex. 1004, page 4 at 'DNA Testing' heading.

164. Leung teaches methods for "detecting aberrantly methylated DNA ... in feces of patients with colorectal cancer or polyps." Ex. 1008 at p. 1070, Abstract (Background).

165. Thus, both Ahlquist and Leung teach that it was routine in the art to determine expression from a human gene by testing nucleic acid for the presence of human DNA having an epigenetic modification. For instance, a person of ordinary skill in the art would be motivated to utilize the teachings of Leung to determine epigenetic modifications in DNA, starting from any of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill because Leung is in the same field as these references. It would be routine to adapt the nucleic acid testing methods of any of any of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with the teachings of Leung in order to investigate epigenetic modifications in genes for their predictive potential of colorectal cancers. Modifying the method of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill to include these teachings of Leung would have required only minor changes to general methods, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 5 based on the

combined teachings of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with De Luca, Olson, and Leung.

166. For the reasons discussed above, it is my opinion that Lapidus '178, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 5 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 5. Therefore, Lapidus '178, De Luca, Olson, and Leung rendered claim 5 obvious.

167. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 5 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 5. Therefore, Lapidus '650, De Luca, Olson, and Leung rendered claim 5 obvious.

168. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 5 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 5. Therefore, Ahlquist, De Luca, Olson, and Leung rendered claim 5 obvious.

169. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 5 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 5. Therefore, O'Neill, De Luca, Olson, and Leung rendered claim 5 obvious.

F. Claims 6-11 Were Obvious

170. Each of claims 6-11 depend, directly or indirectly, from claim 5. Each of claims 6-11 further limit the epigenetic modification of claim 5 or a claim depending from claim 5. In this regard, claim 6 provides "testing the nucleic acid for the presence of human DNA having an epigenetic modification comprises measuring an amount of a methylated human DNA", claim 7 provides "the epigenetic modification comprises aberrant methylation", claim 8 provides "the aberrant methylation comprises hypermethylation", claim 9 provides "the human DNA having an epigenetic modification comprises a gene and/or a promoter region of a gene", claim 10 provides

“the gene is selected from the group consisting of PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFP12, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2, and CDO1”, and claim 11 provides “testing the nucleic acid for the presence of human DNA having an epigenetic modification comprises modifying the nucleic acid with bisulfate ions under conditions wherein unmethylated cytosine is converted to uracil”.

171. Leung teaches “detecting aberrantly methylated DNA ... in feces of patients with colorectal cancer or polyps.” Ex. 1008 at p. 1070, Abstract (Background). Further, Leung teaches that “[f]ecal samples were collected prior to colonoscopy from 20 patients with colorectal cancer, 30 patients with colorectal polyps, and 30 subjects with normal colonic examination. Presence of hypermethylated DNA in 7 tumor-related genes (APC, ATM, hMLH1, sFRP2, HLTF, MGMT, and GSTP1) in stool was analyzed by methylation-specific PCR. *Id.* at p. 1070, Abstract (Methods). Moreover, Leung teaches that “[t]he presence of methylated DNA in stool was detected by methylation-specific PCR (MSP). Briefly, 2 µg of DNA was chemically modified to convert all unmethylated cytosine to uracil by the EZ DNA methylation kit (Zymo Research, Orange, CA). *Id.* at page 1071, second column, first full paragraph.

172. Ahlquist teaches for evaluation of the second stool DNA test (SDT-1), “sequence-specific DNA markers were detected by acrylamide gel electrophoresis, as described by Whitney and colleagues (24); the panel consisted of 3 tumor-specific markers broadly informative for both colorectal cancer and adenomas (25): K-ras mutations, scanning of APC mutator cluster regions, and methylation of the vimentin gene. We used methods described elsewhere to detect mutant Kras (12), APC scanning (25), and vimentin gene methylation (20) assays.” Ex. 1004, page 4 at ‘DNA Testing’ heading.

173. Thus, Leung teaches that it was routine in the art to evaluate the various facets of epigenetic modifications specified by claims 6-11. Ahlquist also suggests certain features of claims 6-11. For instance, a person of ordinary skill in the art would be motivated to utilize the teachings of Leung to determine particular epigenetic modifications in DNA, starting from any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill because Leung is in the same field as these references. It would be routine to adapt the nucleic acid testing methods of any of any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with the teachings of Leung in order to investigate particular epigenetic modifications in genes for their predictive potential of colorectal cancers. For example, Leung teaches that it was routine in the art to detect epigenetic

modification of the SFRP2 gene in feces. In addition, Leung indicates that it well known in the art to use the EZ DNA methylation kit from Zymo Research, which utilizes bisulfate ions for modifying the nucleic acid with under conditions wherein unmethylated cytosine is converted to uracil. Modifying the method of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill to include these teachings of Leung would have required only minor changes to general methods, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 5 based on the combined teachings of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with De Luca, Olson, and Leung.

174. For the reasons discussed above, it is my opinion that Lapidus '178, De Luca, Olson, and Leung collectively taught or suggested each and every element of claims 6-11 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 6-11. Therefore, Lapidus '178, De Luca, Olson, and Leung rendered claims 6-11 obvious.

175. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, Olson, and Leung collectively taught or suggested each and every element of claims 6-11 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 6-11. Therefore, Lapidus '650, De Luca, Olson, and Leung rendered claims 6-11 obvious.

176. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, Olson, and Leung collectively taught or suggested each and every element of claims 6-11 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 6-11. Therefore, Ahlquist, De Luca, Olson, and Leung rendered claims 6-11 obvious.

177. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, Olson, and Leung collectively taught or suggested each and every element of claims 6-11 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 6-11. Therefore, O'Neill, De Luca, Olson, and Leung rendered claims 6-11 obvious.

G. Claim 12 Was Anticipated and Obvious

178. Claim 12 of the '781 Patent provides "[t]he method of claim 4, wherein determining expression from the human gene comprises measuring an amount of RNA expressed from the gene."

179. Lapidus '178 and Lapidus '650 each teach determining expression from the human gene comprises measuring an amount of RNA expressed from the gene. For instance, Lapidus '178 and Lapidus '650 both teach that "DNA or RNA may be isolated from the homogenate using methods known in the art. Subsequent tests may be performed on the isolated DNA and RNA." Ex. 1002 at column 9, lines 47-49; Ex. 1003 at column 8, lines 22-25. Furthermore, Lapidus '178 and '650 both teach that "[n]on-limiting examples of useful known assay protocols include those disclosed in U.S. Pat. Nos. 5,137,806 (detection of sequences in selected DNA molecules), 5,348,855 (assay for nucleic acid sequences), 5,512,441 (detection of mutant alleles), 5,272,057 and 5,380,645 (RFLP analysis), 5,527,676 (detection of p53 gene sequences), 5,330,892 (detection of MCC gene sequences), 5,352,775 (detection of APC gene sequences), 5,532,108 (detection of DCC gene sequences), and in WO96/08514 (monoclonal antibodies against human colon carcinoma-associated antigens), the disclosures of each of which are incorporated by reference herein." Ex. 1002 at column 6, line 58 – column 7, line 2; Ex. 1003 at column 6, lines 15-28. This indicates that RNA was isolated from a fecal sample and an amount of RNA expressed from the gene was determined. Both Lapidus '178 and Lapidus '650 disclose claim 12.

180. Furthermore, any minor differences that may exist between claim 12 and the teachings of Lapidus '178 and Lapidus '650 would have been obvious to a person of ordinary skill in the art at the time of the invention. Indeed, at the time we obtained the Lapidus '178 and Lapidus '650 patents, I envisioned that the methods described in those references could and would involve determining expression from the human gene by measuring an amount of RNA expressed from the gene. Thus, at a minimum, claim 12 was obvious in view of the teachings of Lapidus '178 and/or Lapidus '650.

181. Furthermore, Leung teaches that "COX-2 expression in stool samples was detected by RT-PCR. Messenger RNA was extracted from stool samples by the QIAamp Viral Mini Spin Kit (Qiagen)." Ex. 1008 at page 1071, second column, second full paragraph. Leung

thus teaches it was routine in the art to test RNA from stool to determine expression from the COX-2 gene. A person of ordinary skill in the art would be motivated to utilize the teachings of Leung to determine expression from the human gene comprises measuring an amount of RNA expressed from the gene, starting from any of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill because Leung is in the same field as these references. It would be routine to adapt the nucleic acid testing methods of any of any of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with the teachings of Leung in order to investigate human genes for their predictive potential of colorectal cancers. Modifying the method of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill to include these teachings of Leung would have required only minor changes to general methods, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 12 based on the combined teachings of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with De Luca, Olson., and Leung.

182. In summary, for the reasons discussed above, it is my opinion that Lapidus '178 described each and every element of claim 12. Therefore, Lapidus '178 anticipated claim 12.

183. For the reasons discussed above, it is also my opinion that Lapidus '178 suggested each and every element of claim 12 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '178 rendered claim 12 obvious.

184. For the reasons discussed above, it is also my opinion that Lapidus '650 described each and every element of claim 12. Therefore, Lapidus '650 anticipated claim 12.

185. For the reasons discussed above, it is also my opinion that Lapidus '650 suggested each and every element of claim 12 to those skilled in the art prior to the '781 Patent's filing. Therefore, Lapidus '650 rendered claim 12 obvious.

186. For the reasons discussed above, it is also my opinion that Lapidus '178, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 12 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 12. Therefore, Lapidus '178, De Luca, and Olson rendered claim 12 obvious.

187. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 12 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 12. Therefore, Lapidus '650, De Luca, and Olson rendered claim 12 obvious.

188. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 12 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 12. Therefore, Ahlquist, De Luca, and Olson rendered claim 12 obvious.

189. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 12 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 12. Therefore, O'Neill, De Luca, and Olson rendered claim 12 obvious.

H. Claim 13 Was Obvious

190. Claim 13 of the '781 Patent provides "[t]he method of claim 12, wherein measuring an amount of RNA expressed from the gene comprises reverse transcriptase polymerase chain reaction (RT-PCR)."

191. Leung teaches that "COX-2 expression in stool samples was detected by RT-PCR. Messenger RNA was extracted from stool samples by the QIAamp Viral Mini Spin Kit (Qiagen)." Ex. 1008 at page 1071, second column, second full paragraph. Leung thus teaches it was routine in the art to test RNA from stool to determine expression from the COX-2 gene. A person of ordinary skill in the art would be motivated to utilize the teachings of Leung to measure an amount of RNA expressed from the gene via RT-PCR, starting from any of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill because Leung is in the same field as these references. It would be routine to adapt the nucleic acid testing methods of any of any of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with the teachings of Leung in order to investigate RNA via RT-PCR because it was known at the time of filing that RT-PCR provided means to evaluate RNA expression. Modifying the method of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill to

include these teachings of Leung would have required only minor changes to general methods regarding RT-PCR, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 13 based on the combined teachings of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with De Luca, Olson, and Leung.

192. For the reasons discussed above, it is my opinion that Lapidus '178, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 13 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 13. Therefore, Lapidus '178, De Luca, Olson, and Leung rendered claim 13 obvious.

193. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 13 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 13. Therefore, Lapidus '650, De Luca, Olson, and Leung rendered claim 13 obvious.

194. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 13 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 13. Therefore, Ahlquist, De Luca, Olson, and Leung rendered claim 13 obvious.

195. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, Olson, and Leung collectively taught or suggested each and every element of claim 13 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 13. Therefore, O'Neill, De Luca, Olson, and Leung rendered claim 13 obvious.

I. Claim 14 Was Obvious

196. Claim 14 of the '781 Patent provides "[t]he method of claim 3, wherein testing for an amount of a blood protein in the removed portion comprises testing for a concentration of hemoglobin in the removed portion."

197. Lapidus '178 and Lapidus '650 each teach testing for an amount of a blood protein in the removed portion such as testing for a concentration of hemoglobin. For instance, Lapidus '178 and '650 both teach that a removed portion such as a cross-sectional stool sample or the like "may be homogenized by known methods to distribute cells and cellular debris throughout the sample. An assay then is performed on the homogenate, or an extract of the homogenate, to detect the presence of cells and/or cellular debris in the sample. The assay may be any one or a combination of histological cellular assays, antibody based immunoassays (or other formats) designed to detect the presence of a molecule characteristic of transformation such as a protein, or DNA-based assays for detecting mutations or genetic characteristics indicative of colorectal cancer." Ex. 1003 at column 6, lines 1-11. Furthermore, Lapidus '178 and Lapidus '650 each teach that "[i]f a fecal occult blood assay is to be performed, the buffer may contain chemical compounds which react with blood to produce a color, the intensity of which can be measured. Buffers useful for detecting the presence of fecal occult blood are known in the art." *Id.* at column 8, lines 13-18.

198. De Luca discloses "collection of faeces samples, particularly for laboratory immunological tests, for qualitative or quantitative determination of one or more analytes, such as haemoglobin...". Ex. 1006 at [0001].

199. Ahlquist discloses for occult blood testing that "[t]he manufacturer that developed the Hemoccult and HemoccultSensa cards, without rehydration, trained technicians on-site at the Mayo Clinic. As recommended by the manufacturer, the technicians added the catalyst solution to cards stored at ambient temperature within 48 to 72 hours of collection. We defined a spreading (enlarging) blue color in 60 seconds in any window of the cards as a positive result and any other result as negative." Ex. 1004 at page 5, at heading 'Occult Blood Testing.' It is well known to the skilled artisan that Hemoccult and HemoccultSensa tests test for the presence of hemoglobin.

200. O'Neill discloses "a method and apparatus for removing cellular material from faecal stools and a container for collecting faecal stools." Ex. 1005 at page 1, lines 5-7. O'Neill

discloses that “[e]xfoliated epithelial cells consist of cellular components, such as DNA, RNA and proteins, which potentially provide a multiplicity of approaches to identifying and monitoring unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis. The facile recovery of such cells makes possible the application of a wide variety of test procedures for qualitative and/or quantitative analysis of, for example, DNA alterations, gene expression and enzyme activity.” *Id.* at page 1, lines 15-24. It is well known to the skilled artisan that such proteins and associated enzyme activity would include testing for the presence of hemoglobin in order to identify unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis.

201. A person of ordinary skill in the art would be motivated to utilize the teachings of De Luca to test for an amount of a blood protein in the removed portion such as testing for a concentration of hemoglobin, starting from any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill because De Luca is in the same field as these references. It would be routine to adapt the protein and enzyme testing methods of any of any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with the teachings of De Luca in order to investigate concentrations of hemoglobin. Modifying the method of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill to include these teachings of De Luca would have required only minor changes to general methods regarding hemoglobin, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 14 based on the combined teachings of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with De Luca and Olson.

202. For the reasons discussed above, it is my opinion that Lapidus ‘178, De Luca, and Olson collectively taught or suggested each and every element of claim 14 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 14. Therefore, Lapidus ‘178, De Luca, and Olson rendered claim 14 obvious.

203. For the reasons discussed above, it is also my opinion that Lapidus ‘650, De Luca, and Olson collectively taught or suggested each and every element of claim 14 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to combine those

teachings to arrive at the subject matter of claim 14. Therefore, Lapidus '650, De Luca, and Olson rendered claim 14 obvious.

204. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, and Olson collectively taught or suggested each and every element of claim 14 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 14. Therefore, Ahlquist, De Luca, and Olson rendered claim 14 obvious.

205. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 14 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 14. Therefore, O'Neill, De Luca, and Olson rendered claim 14 obvious.

J. Claim 15 Was Obvious

206. Claim 15 of the '781 Patent provides "[t]he method of claim 14, wherein testing for the concentration of hemoglobin comprises immunochemical detection of hemoglobin."

207. Lapidus '178 and Lapidus '650 each teach testing for the concentration of hemoglobin comprises immunochemical detection of hemoglobin. For instance, Lapidus '178 and '650 both teach that a removed portion such as a cross-sectional stool sample or the like "may be homogenized by known methods to distribute cells and cellular debris throughout the sample. An assay then is performed on the homogenate, or an extract of the homogenate, to detect the presence of cells and/or cellular debris in the sample. The assay may be any one or a combination of histological cellular assays, antibody based immunoassays (or other formats) designed to detect the presence of a molecule characteristic of transformation such as a protein, or DNA-based assays for detecting mutations or genetic characteristics indicative of colorectal cancer." Ex. 1003 at column 6, lines 1-11. Furthermore, Lapidus '178 and Lapidus '650 each teach that "[i]f a fecal occult blood assay is to be performed, the buffer may contain chemical compounds which react with blood to produce a color, the intensity of which can be measured. Buffers useful for detecting the presence of fecal occult blood are known in the art." *Id.* at column 8, lines 13-18.

208. De Luca discloses “collection of faeces samples, particularly for laboratory immunological tests, for qualitative or quantitative determination of one or more analytes, such as haemoglobin...”. Ex. 1006 at [0001].

209. Ahlquist discloses for occult blood testing that “[t]he manufacturer that developed the Hemoccult and HemoccultSensa cards, without rehydration, trained technicians on-site at the Mayo Clinic. As recommended by the manufacturer, the technicians added the catalyst solution to cards stored at ambient temperature within 48 to 72 hours of collection. We defined a spreading (enlarging) blue color in 60 seconds in any window of the cards as a positive result and any other result as negative.” Ex. 1004 at page 5, at heading ‘Occult Blood Testing.’ It is well known to the skilled artisan that Hemoccult and HemoccultSensa test for the presence of hemoglobin.

210. O’Neill discloses “a method and apparatus for removing cellular material from faecal stools and a container for collecting faecal stools.” Ex. 1005 at page 1, lines 5-7. O’Neill discloses that “[e]xfoliated epithelial cells consist of cellular components, such as DNA, RNA and proteins, which potentially provide a multiplicity of approaches to identifying and monitoring unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis. The facile recovery of such cells makes possible the application of a wide variety of test procedures for qualitative and/or quantitative analysis of, for example, DNA alterations, gene expression and enzyme activity.” *Id.* at page 1, lines 15-24. It is well known to the skilled artisan that such proteins and associated enzyme activity would include testing for the presence of hemoglobin in order to identify unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis.

211. A person of ordinary skill in the art would be motivated to utilize the teachings of De Luca to test for the concentration of hemoglobin using immunochemical detection of hemoglobin, starting from any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill because De Luca is in the same field as these references. Further, Lapidus ‘178 and Lapidus ‘650 expressly teach that antibody based immunoassays can be used on fecal samples. It would be routine to testing methods of any of any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with the teachings of De Luca in order to use immunochemical detection of hemoglobin because De Luca (and Ahlquist) indicates that such evaluations are more effective to identify hemoglobin in fecal samples compared to methods such as Hemoccult and HemoccultSensa. A skilled person would

have a reasonable expectation of success to use immunochemical detection of hemoglobin because De Luca that such hemoglobin evaluations are routine in the art and each of Lapidus '178, Lapidus '650, Ahlquist, and O'Neill suggest that immunochemical detection of hemoglobin can be used for the given patient population. As such, modifying the method of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill to include these teachings of De Luca would have required only minor changes to general methods, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claim 15 based on the combined teachings of Lapidus '178, Lapidus '650, Ahlquist, or O'Neill with De Luca and Olson.

212. For the reasons discussed above, it is my opinion that Lapidus '178, De Luca, and Olson collectively taught or suggested each and every element of claim 15 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 15. Therefore, Lapidus '178, De Luca, and Olson rendered claim 15 obvious.

213. For the reasons discussed above, it is also my opinion that Lapidus '650, De Luca, and Olson collectively taught or suggested each and every element of claim 15 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 15. Therefore, Lapidus '650, De Luca, and Olson rendered claim 15 obvious.

214. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, and Olson collectively taught or suggested each and every element of claim 15 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 15. Therefore, Ahlquist, De Luca, and Olson rendered claim 15 obvious.

215. For the reasons discussed above, it is also my opinion that O'Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 15 to those skilled in the art prior to the '781 Patent's filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 15. Therefore, O'Neill, De Luca, and Olson rendered claim 15 obvious.

K. Claims 16-20 Were Obvious

216. Each of claims 16-20 depend from claim 14. Each of claims 16-20 further specify that “the removed portion of the fecal sample is considered positive for the presence of blood” at a certain concentration of hemoglobin detected in the removed portion. For instance, claim 16 indicates positivity when the concentration of hemoglobin detected in the removed portion is at least 5 ng/ml. Claims 17, 18, 19, and 20 are similar in language but specify concentrations of at least 10 ng/ml, at least 20 ng/ml, at least 50 ng/ml, and at least 200 ng/ml, respectively.

217. As explained above, it is my opinion that combinations of Lapidus ‘178, Lapidus ‘650, Ahlquist, O’Neill, De Luca, and Olson collectively taught or suggested each and every element of claim 15 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claim 15.

218. Hoepffner discloses that “the sensitivity of [various] tests to detect faecal [hemoglobin] was 37.0%, 74.0% and 77.7% for the Guaiac-based test, the bedside IFOBT and the ELISA (cut-off **<10 ng/mL**) respectively.” Ex. 1009, p. 148, second column, first full paragraph (emphasis added). . Hoepffner thus teaches it was routine in the art to assign a concentration of hemoglobin to indicate when a fecal sample is considered positive for the presence of blood. For instance, Hoepffner teaches that a sample is positive for blood when a concentration of hemoglobin is at least 10 ng/ml. Thus, a person skilled in the art would understand that Hoepffner suggests any concentration at or above 10 ng/ml (e.g., 20 ng/ml, 50 ng/ml, and 200 ng/ml) would also be considered positive for the presence of blood.

219. A person of ordinary skill in the art would be motivated to utilize the teachings of Hoepffner to determine a concentration of hemoglobin that could be utilized as a “cut-off” for the presence of blood in a fecal sample. Starting from any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill, a person of ordinary skill in the art would be motivated to combine these teachings because Hoepffner is in the same field as these references. It would be routine to adapt the methods of protein (e.g., hemoglobin) detection any of any of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with the teachings of Leung in order to assign a cut-off value according to Hoepffner to assist in diagnosing colorectal cancers. Furthermore, a skilled person would be motivated to further modify the teachings of Hoepffner in order to reduce the “cut-off” value of hemoglobin concentration to be as low as possible. It would be routine to modify the values to

lower concentrations in order to make the hemoglobin test more sensitive, thus being able to identify more patients that were at risk for colorectal cancer. A skilled person would have a reasonable expectation of success to determine a concentration of hemoglobin that could be utilized as a “cut-off” for the presence of blood in a fecal sample because Hoepffner teaches that such determinations are routine in the art. Modifying the method of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill to include these teachings of Hoepffner would have required only minor changes to general methods, all of which were well within the knowledge and technical grasp of a person of ordinary skill in the art, who would therefore have a reasonable expectation of success. It also would have been a predictable use of the prior art according to their established functions and yielded predictable results. As such, one skilled in the art would have found it obvious to arrive at claims 16-20 based on the combined teachings of Lapidus ‘178, Lapidus ‘650, Ahlquist, or O’Neill with De Luca, Olson, and Hoepffner.

220. For the reasons discussed above, it is my opinion that Lapidus ‘178, De Luca, Olson, and Hoepffner collectively taught or suggested each and every element of claims 16-20 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 16-20. Therefore, Lapidus ‘178, De Luca, Olson, and Hoepffner rendered claims 16-20 obvious.

221. For the reasons discussed above, it is also my opinion that Lapidus ‘650, De Luca, Olson, and Hoepffner collectively taught or suggested each and every element of claims 16-20 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 16-20. Therefore, Lapidus ‘650, De Luca, Olson, and Hoepffner rendered claims 16-20 obvious.

222. For the reasons discussed above, it is also my opinion that Ahlquist, De Luca, Olson, and Hoepffner collectively taught or suggested each and every element of claims 16-20 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to combine those teachings to arrive at the subject matter of claims 16-20. Therefore, Ahlquist, De Luca, Olson, and Hoepffner rendered claims 16-20 obvious.

223. For the reasons discussed above, it is also my opinion that O’Neill, De Luca, Olson, and Hoepffner collectively taught or suggested each and every element of claims 16-20 to those skilled in the art prior to the ‘781 Patent’s filing and that it would have been obvious to

combine those teachings to arrive at the subject matter of claims 16-20. Therefore, O'Neill, De Luca, Olson, and Hoepffner rendered claims 16-20 obvious.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001.

Dated: May 22, 2023

/Anthony P. Shuber/

Mr. Anthony P. Shuber, MS

EXHIBITS

- Exhibit 1001: U.S. Patent No. 11,634,781 (“the ‘781 Patent”)
- Exhibit 1002: U.S. Patent No. 5,952,178 to Lapidus et al. (“Lapidus ‘178”)
- Exhibit 1003: U.S. Patent No. 5,741,650 to Lapidus et al. (“Lapidus ‘650”)
- Exhibit 1004: Ahlquist et al., “Stool DNA and Occult Blood Testing for Screen Detection of Colorectal Neoplasia,” *Ann Intern Med.*, 149(7):441-W81 (published in final form October 7, 2008) (“Ahlquist”)
- Exhibit 1005: Patent Cooperation Treaty (PCT) Publication No. WO 2005/014154 to O’Neill (“O’Neill”)
- Exhibit 1006: European Patent Application Publication No. EP1366715A1 to De Luca et al. (“De Luca”)
- Exhibit 1007: Olson et al., “DNA Stabilization Is Critical for Maximizing Performance of Fecal DNA-Based Colorectal Cancer Tests,” *Diagn Mol Pathol*, 14:183–191 (published September 2005) (“Olson”)
- Exhibit 1008: Leung et al., “Detection of Hypermethylated DNA or Cyclooxygenase-2 Messenger RNA in Fecal Samples of Patients With Colorectal Cancer or Polyps,” *Am J Gastroenterol*, 102:1070–1076 (2007) (“Leung”)
- Exhibit 1009: Hoepffner et al., “Comparative evaluation of a new bedside faecal occult blood test in a prospective multicentre study,” *Aliment Pharmacol Ther*, 23, 145–154 (published 2006) (“Hoepffner”)
- Exhibit 1010: U.S. Patent No. 4,859,610 to Maggio (“Maggio”)
- Exhibit 1011: U.S. Patent No. 5,196,167 to Guadagno et al. (“Guadagno”)
- Exhibit 1012: U.S. Patent No. 5,137,806 to LeMaistre et al. (“LeMaistre”)
- Exhibit 1013: U.S. Patent No. 5,527,676 to Vogelstein et al. (“Vogelstein”)

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

Jeff Olson, BS, Duncan H. Whitney, PhD, Kristine Durkee, PhD, and Anthony P. Shuber, MS

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

(*Diagn Mol Pathol* 2005;14:183–191)

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, 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			
GP34	36 HR B	58			2770			2750			11			
	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)			
Sample	Time Point	DIA-D 200	DIA-E 200	DIA-X 200	DIA-Y 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.

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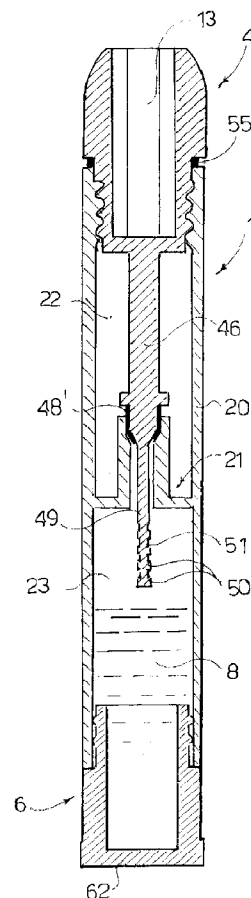
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(54) Extraction tube for collection of faeces samples

(57) An extraction tube (1) is described for the collection of faeces samples, comprising a container body (2) hollow on the inside and open at the top to receive a top cap (4) provided with a threaded small rod (49) for collection of faeces samples. When the top cap (4) is applied to the top end of the container body (2), the threaded small rod protrudes axially into the container body. The container body (2) is also open at the bottom to receive a bottom cap (6). Said extraction tube is employed as a primary collection tube to be inserted in the sample-holder plate of automatic analysers for clinical chemistry and immunological tests.

FIG. 3

Description

[0001] The present invention refers to an extraction and sampling device for collection of faeces samples, particularly for laboratory immunological tests, for qualitative or quantitative determination of one or more analytes, such as haemoglobin, *Helicobacter pylori* and the like.

[0002] At present, the following two methods, among others, are known for testing for analytes present in faeces samples: the chemical method wherein a defined amount of the sample collected is added to a reagent constituting an active principle which reacts chemically with the analyte under examination giving rise to a chromogenic reaction indicative of the presence of the analyte and the immunological or immunochromographic method wherein the sample collected is dissolved in a buffer solution which is dropped onto a diagnostic device containing an absorbent membrane treated with specific antibodies bound to coloured substances which fix the analytes under examination giving rise to a coloured sign on the membrane.

[0003] These tests are done manually by the operator and are of a qualitative type; that is to say, they reveal the presence or absence of an analyte.

[0004] To carry out sampling of the faeces an extraction tube comprising a cylindrical container with a screw closure cap is used. In the lower inside surface of the cap there is mounted a screw small rod which protrudes axially downwards therefrom. The screw small rod has a threaded portion in its free end, destined for collection of faeces sample. In fact, when the small rod is inserted into the faeces, faeces samples are retained in its thread.

[0005] When the cap is screwed onto the mouth of the container, the screw small rod is partially immersed inside the container, which is suitably filled with a buffer solution having a suitable pH. In this manner the collected faeces sample detaches from the thread of the screw small rod and is dispersed in the buffer solution.

[0006] In the upper outside surface of the cap a breakable tip protruding axially and upward therefrom is provided. Breakage of the tip forms an outlet aperture in the cap communicating with the inside of the container. In this manner the cap acts as a dropper allowing the buffer solution - wherein faeces sample is dissolved - to drop through the outlet aperture.

[0007] During the sampling operation the patient must unscrew the cap from the container, plunge the screw small rod into the faeces so that faeces samples are retained in the thread of the screw small rod, remove with the aid of a cotton wool ball - if possible - the excess faeces accumulated on the small rod outside the thread, and lastly screw the screw cap into the container to immerse the screw small rod in the buffer solution.

[0008] Subsequently, to perform the test, the tip is broken and the buffer solution containing the sample is dropped onto a suitable diagnostic device which con-

tains a nitrocellulose membrane treated with specific antibodies to the analyte (for example haemoglobin) under examination.

[0009] Said collection tube presents some drawbacks. In fact the operation of removing the excess faeces with cotton wool is quite awkward for the user. Furthermore, the outlet aperture of the dropper often becomes obstructed with consequent problems in releasing the solution. Also, the tip of the cap which closes the outlet aperture can break accidentally during handling and/or transport with the result of involuntary release of the solution containing biologically hazardous material.

[0010] The drawback of removal of excess faeces from the screw small rod is solved by known extraction tubes, which provide a dividing partition in the form of an annular flange disposed in the container above the level of the buffer solution. Said dividing flange has a central hole wherein the screw small rod is inserted so that the excess faeces is retained above the dividing flange and does not fall into the buffer solution.

[0011] In this case, the tip of the outlet aperture is disposed in the part of the container opposite the mouth that receives the cap of the screw small rod. Another already known solution provides a device similar to that described above wherein a second screw cap disposed to protect the tip is provided, to prevent accidental breakage thereof.

[0012] However, in extraction tubes of the prior art the problem of occlusion of the outlet aperture of the dropper nevertheless remains. Furthermore said extraction tubes have been designed for manual tests.

[0013] For use in automated systems, the buffer solution containing the dissolved faeces samples must be transferred to special sample-holder tubes which are labelled with identification codes and loaded into an automatic analyser which, in a per se known manner, performs the test.

[0014] It is apparent that said operation of transferring the buffer solution with faeces samples to the sample-holder tube of automatic analysers is quite tiresome for the operator concerned and involves an excessive waste of time.

[0015] It is an object of the present invention to overcome the prior art drawbacks by providing an extraction and sampling tube for the collection of faeces samples that is practical for the patient and simple to use for the laboratory technician.

[0016] Another object of the present invention is to provide an extraction and sampling tube for the collection of faeces samples that is versatile and suitable to be applied directly to different test systems present on the market, such as automatic clinical chemistry and immunological test analysers.

[0017] Yet another object of the present invention is to provide an extraction and sampling tube for faeces collection that is economical and simple to make.

[0018] These objects are achieved according to the invention with the characteristics listed in appended in-

dependent claim 1.

[0019] Advantageous embodiments of the invention are apparent from the dependent claims. The extraction tube for collection of faeces samples, according to the invention, comprises a container body hollow on the inside for containing a buffer solution and faeces samples, a top cap applied to the top end of the container body and provided with a threaded small rod for sampling of faeces samples and a bottom cap applied removably to the bottom end of the container body.

[0020] The main characteristic of the present invention is that said extraction tube can be used directly for automatic tests.

[0021] In the extraction tube according to the invention the breakable tip provided by the prior art extraction tubes is eliminated and, consequently, all the drawbacks due to the breakable tip are eliminated.

[0022] Furthermore, the upper part of the top cap of the extraction tube according to the invention is advantageously substantially dome-shaped, similarly to the bottom of a primary sampling tube, which makes its use possible on automatic clinical chemistry and immunological test analysers. The diameter of the container body of the extraction device according to the invention is also substantially equal to the diameter of the sample tubes routinely used in laboratories. Consequently the extraction tube according to the invention can be used directly on automatic clinical chemistry and immunological test analysers, without the need of transferring the contents thereof to special test tubes suitable to be positioned in the sample-holder plate of said automatic analysers.

[0023] Further characteristics of the invention will be made clearer by the following detailed description, referring to a purely exemplary and therefore non-limiting embodiment thereof, illustrated in the appended drawings, in which:

Figure 1 is an exploded front view illustrating the extraction tube for collection of faeces samples according to the invention;

Figure 2 is an axial sectional view along the plane of section plane II-II of Figure 1;

Figure 3 is an axial sectional view illustrating the extraction tube according to the invention assembled and with a buffer solution therein;

Figure 4 is an axial sectional view illustrating the extraction tube according to the invention, ready for use in a support of an automatic machine for clinical chemistry and immunological tests, wherein the support is shown broken off.

[0024] The extraction tube for collection of faeces samples according to the invention, designated as a whole with reference numeral 1, is described with the aid of the figures.

[0025] In particular, with reference to Figures 1 and 2 the extraction tube 1 which comprises a container 2, a

top cap 4 and a bottom cap 6 is shown exploded.

[0026] The container 2 comprises a substantially cylindrical body 20, hollow on the inside and open at the top and at the bottom. Inside the body 20 of the container, in an intermediate position, a dividing partition 21 is provided which divides the inside of the container 2 into a top chamber 22 and a bottom chamber 23.

[0027] The dividing partition 21 comprises an annular flange 24 which protrudes radially inwards. A cylindrical tang 25 protrudes axially upward from the top of the annular flange 24. The dividing partition 21 has an axial through hole 26 which passes axially through the cylindrical tang 25 and the annular flange 24. The hole 26 has in its upper part a substantially tapered portion 26' with an increasing diameter with respect to the lower part.

[0028] In the inside surface of the container body 20, in the upper end of the top chamber 22, an internal thread 27 is provided.

[0029] The top cap 4 comprises a body consisting of a substantially cylindrical middle part 40 with an outside diameter substantially equal to the outside diameter of the container body 20, a substantially dome-shaped top part 41 disposed above the middle part and a substantially cylindrical bottom part 42, disposed beneath the middle part and having an outside diameter smaller than that of the middle part and slightly smaller than the inside diameter of the container body 20.

[0030] An upwardly open blind hole 43, hexagonal in shape in cross section, is provided axially in the body of the cap 4.

[0031] Knurling 44 consisting of a plurality of longitudinal ribs and grooves is provided in the outside surface of the middle part 40 of the body of the top cap 44.

[0032] An annular seal 55 is provided between the knurled part 44 and the threaded part 45 of the top cap.

[0033] An external thread 45 able to engage, in a screwing relationship, with the internal thread 27 of the container body is provided in the outer surface of the central bottom part 42.

[0034] A stem 46 having a smaller diameter than the bottom part 42 protrudes axially downward from the bottom part 42 of the top cap. At the bottom end of the stem 46 a collar or annular flange 47 which protrudes radially from the stem 46 is provided. The collar 47 has an outside diameter substantially equal to the outside diameter of the tang 25 of the dividing partition of the container 2.

[0035] A tang 48 with a smaller outside diameter than the collar 47 is provided beneath the collar 47. A seal 48' able engage sealably in the tapered part 26' of the hole 26 of the dividing partition 21 is disposed around the tang 48. A small rod 49 with a smaller diameter than the tang 48 and the hole 26 in the dividing partition 21 is provided axially beneath the tang 48.

[0036] A thread formed by a plurality of collars 50 disposed spaced apart from each other so as to form annular spaces 51 between said collars 50 is provided in

the bottom portion of the small rod 49.

[0037] The bottom cap 6 comprises a body consisting of a middle cylindrical part 60 having an outside diameter substantially equal to the outside diameter of the container 2, a top cylindrical part 61 having a slightly smaller outside diameter with respect to the inside diameter of the container body 20 and a disk-shaped bottom part 62 having an outside diameter slightly greater than the outside diameter of the central part.

[0038] An upwardly open blind hole 63 is provided axially in the body of the bottom cap 6. Knurling 64 consisting of a plurality of longitudinal ribs and grooves is provided in the outer surface of the middle part 60 of the bottom cap 6.

[0039] Annular collars 65 which protrude radially outward so that the top part 61 of the bottom cap can engage by pressure in the bottom part 22 of the container are provided in the outer surface of the top part 61.

[0040] Assembly of the extraction tube 1 is described below with reference to Figure 3. The external thread 45 of the top cap 3 is screwed into the internal thread 27 of the container.

[0041] In this manner the small rod 49 of the top cap 4 enters the axial hole 26 of the dividing partition 21, until the seal 48' of the tang of the small rod settles tightly in the tapered part 26' of the hole 26 in the dividing partition 21 and at the same time the annular seal 55 of the top cap is disposed tightly on the upper edge of the container body 20. In this situation, the threaded part 50 of the small rod 49 is situated inside the bottom chamber 23 of the container, the seal 48' of the tang 48 of the small rod 49 is housed sealably inside the tapered part 26' of the hole 26 of the dividing partition and thus the top chamber 22 of the container is perfectly separated from the bottom chamber 23 of the container.

[0042] At this point a buffer solution 8 is introduced through the bottom opening of the container body 2 and then the bottom cap 6 is applied. The buffer solution 8 can be, for example, a solution with a pH between 7 and 9 with specific stabilizers. It should be noted that, by virtue of the presence of the dividing partition 21 closed by the seal 48' of the tang 48 of the stem of the top cap, the buffer solution 8 cannot pass from the bottom chamber 23 to the top chamber 22 of the container.

[0043] As shown in Figure 3, when the extraction tube 1 is in the upright position, that is to say when the bottom part 62 of the bottom cap 6 rests on a horizontal plane, the buffer solution 8 is partially inside the blind hole 63 of the bottom cap and partially in the bottom chamber 23 of the container, whilst the threaded part 50 of the small rod 49 of the top cap is not immersed in the buffer solution 8.

[0044] The user, when has to collect a faeces sample, unscrews the top cap 4 and plunges the small rod 49 into the faeces. Consequently, on extracting the small rod 49 from the faeces, faeces samples are retained in the annular spaces 51 in the thread 50 of the small rod 49, and also excess faeces remain around the small rod

49.

[0045] Subsequently the user screws the top cap 4 into the container 2. As a result the small rod 49 passes through the hole 26 in the dividing partition and the excess faeces are retained by the upper edge of the tang 25 of the dividing partition and thus remain in the top chamber 22. On the other hand, the faeces required for the analysis is retained in the annular spaces 51 of the thread 50 of the small rod.

[0046] By shaking the extraction tube 1, the buffer solution 8 immerses the threaded part 50 of the screw small rod and, as a result, the faeces samples are dispersed in the buffer solution. At this point the extraction tube 1 can be used as a primary sampling tub to be inserted in the sample-holder plate of automatic clinical chemistry and immunological test analysers.

[0047] As shown in Figure 4, in the extraction tube 1, in the upturned position, the buffer solution 8 occupies the bottom chamber 23 and totally immerses the threaded part 50 of the small rod 49. It should be noted that, even in this situation, due to the presence of the dividing partition 21 which is closed by the seal 48' of the tang of the small rod, the buffer solution 8 cannot pass from the bottom chamber 23 to the top chamber 22 of the container.

[0048] At this point the extraction tube 1 is positioned in a special housing 9 of an automatic machine for immunological tests and then the bottom cap 6 is removed so as to allow removal of the buffer solution 8 containing the faeces samples by a probe of the automatic machine.

[0049] It should be noted that the dome shape 41 of the upper part of the top cap 4 is designed to be substantially similar to the bottom of test tubes typically used in automatic analysers for clinical chemistry and immunological tests, to allow insertion of the extraction tube 1 inside the housing 9 of the automatic test machine.

[0050] Numerous changes and variations of detail within the reach of a person skilled in the art can be made to the present embodiment of the invention, without thereby departing from the scope of the invention as set forth in the appended claims.

Claims

1. An extraction tube (1) for collection of faeces samples comprising:

- a container body (2), hollow on the inside, open at the top, and able to receive a buffer solution,
- a top cap (4) provided with a threaded small rod (49) for collection of faeces samples, said threaded small rod protruding axially inside the container body, when the top cap (4) is applied to the top end of the container body (2),
- a dividing partition (21) provided, in an interme-

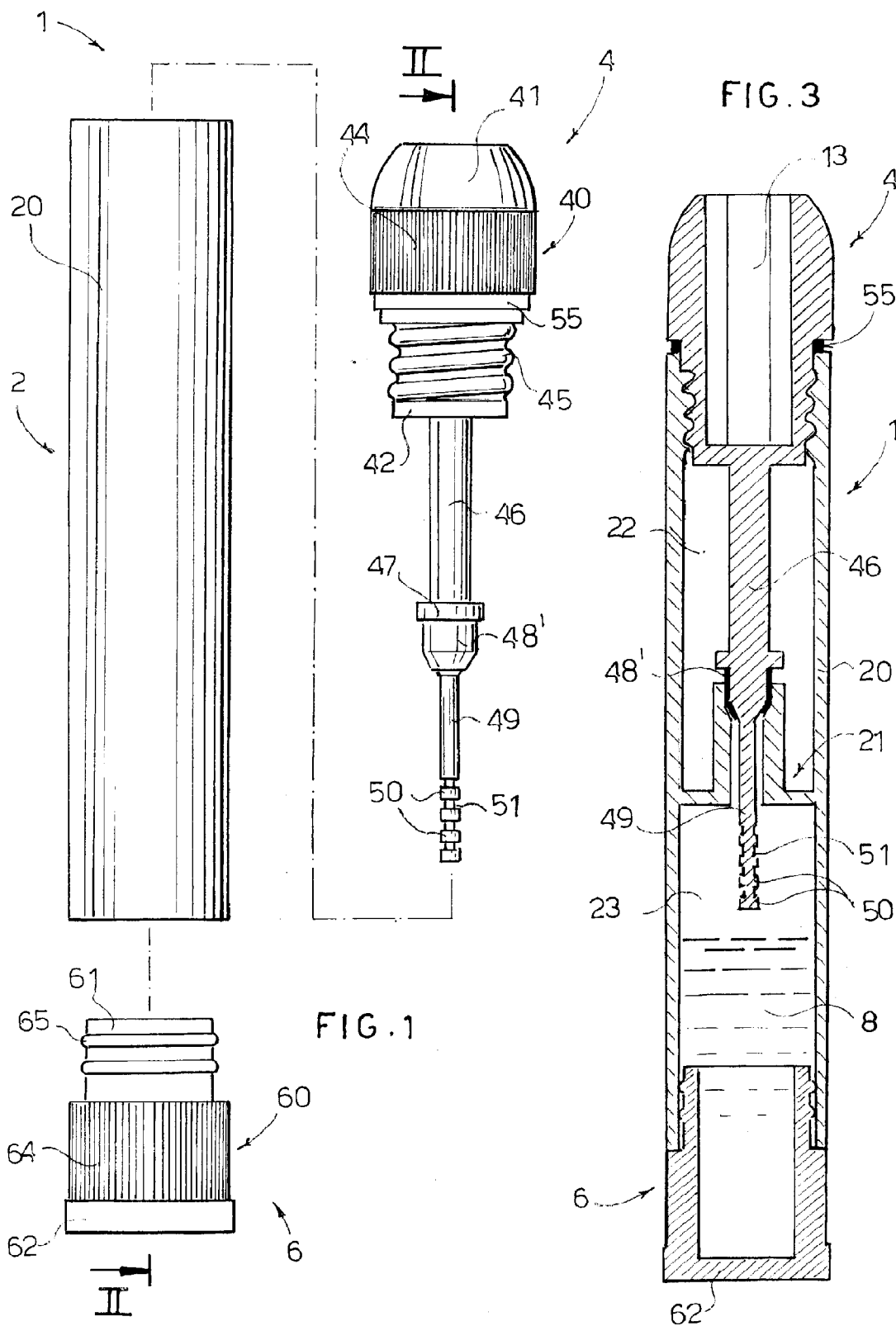
diate position, inside said container body (2) so as to separate a top chamber (22) from a bottom chamber (23) inside said container body, said dividing partition (21) having an axial hole (26) suitable to allow the passage of said threaded small rod (49), so as to retain the excess faeces in said top chamber (22) and allow the passage of the threaded part of the small rod into said bottom chamber (23),

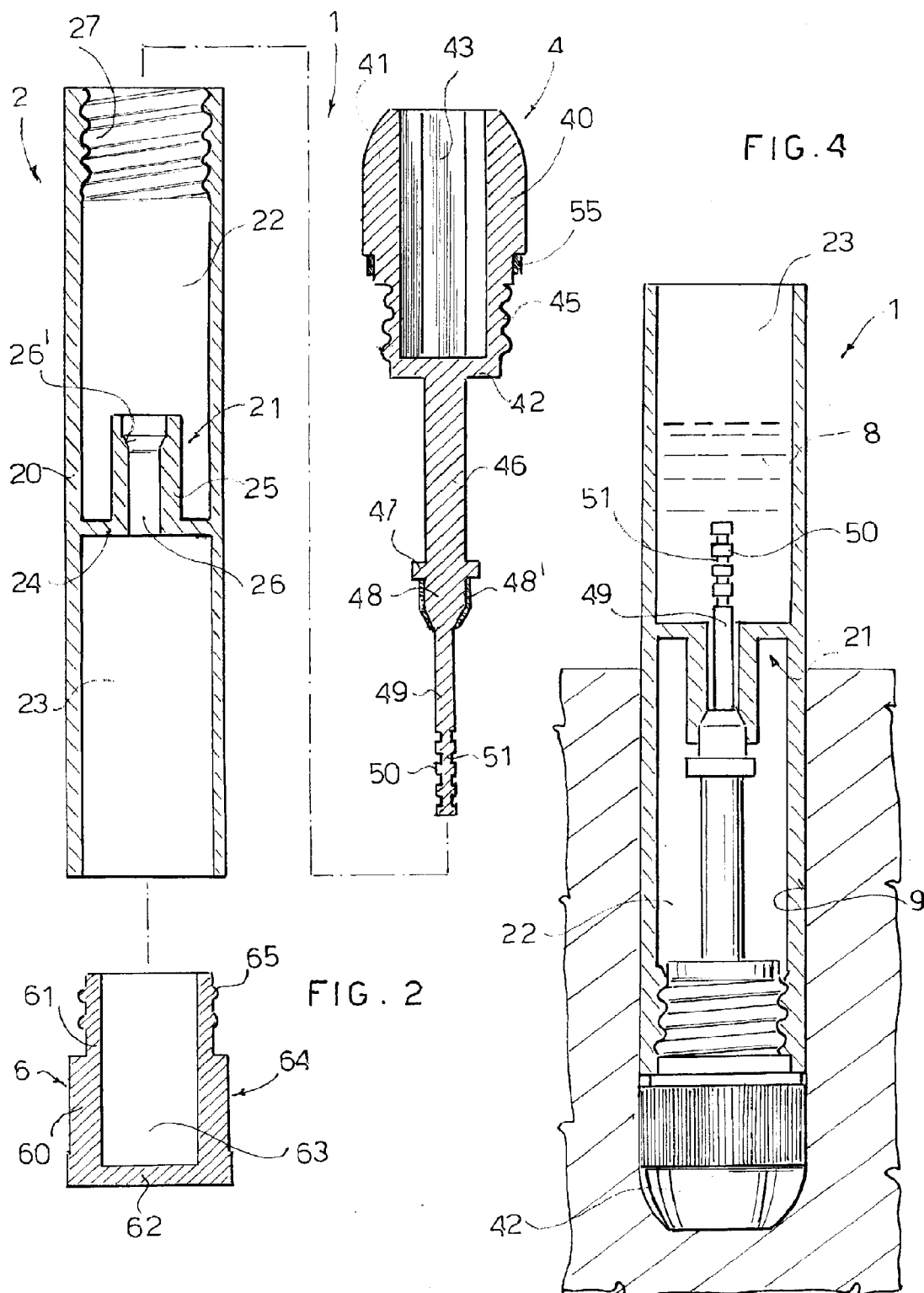
characterised in that said container body is open at the bottom and provided with a bottom cap (6) which can be applied movably to the bottom end of the container body, so that said extraction tube can be used directly as a primary sampling tube to be inserted in a sample-holder plate of automatic analysers, following removal of said bottom cap (6) and overturning of said container body (2) for positioning in a housing (9) of the automatic analyser, with the top cap (4) facing downward.

2. An extraction tube (1) according to claim 1, **characterised in that** said top cap (4) has an upper portion (41) destined to be disposed outside said container body (2), said upper portion (41) having a substantially domed shape similar to the bottom of a test tube normally used in the sample-holder plate of automatic analysers.
3. An extraction tube according to claim 1 or 2, **characterised in that** said top cap (4) comprises a stem (46) ending in an annular flange (47) beneath which is provided a cylindrical tang (48) surrounded by a seal (48') beneath which said threaded small rod (49) protrudes axially, and said dividing partition (21) comprising an annular flange (24) from which a cylindrical tang (25) protrudes axially upward so that when said top cap (4) is applied to the top end of the container body, the seal 48' of the cylindrical tang (48) of the stem (46) of the top cap is housed sealably inside a tapered part (26') of said hole (26) in the dividing partition.
4. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said top cap (4) comprises a bottom portion (42) having an external thread (45) able to engage in an internal thread (27) provided in the top inner part of said container body (2).
5. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said top cap (4) comprises a middle portion (40) having knurling (42) consisting of a plurality of longitudinal ribs and grooves.
6. An extraction tube (1) according to claims 4 and 5, **characterised in that** between said knurled part

(44) of the top cap and said threaded part (45) of the top cap there is provided an annular seal (55) able to abut sealably on the top edge of said container body 2.

7. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said top cap (4) has a blind hole (43) axially open at the top to the outside of the container body, and hexagonal in shape in cross section.
8. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said bottom cap (6) has a top portion (61) with a plurality of annular collars (65) protruding radially outwards, to be able to be applied by pressure into the bottom part of said container body (2).
9. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said bottom cap (6) comprises a middle portion (60) with knurling (64) consisting of a plurality of longitudinal ribs and grooves.
10. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said bottom cap (6) has a blind hole (63) axially open at the top to the inside of the container body (2).
11. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said threaded small rod (49) has in its bottom portion a thread consisting of a plurality of concentric collars (50) so as to define a plurality of annular spaces (51) therebetween, able to retain the sampled faeces.







European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 03 01 1896

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	EP 0 727 653 A (WAKO PURE CHEM IND LTD) 21 August 1996 (1996-08-21) * column 3, line 17 - line 38 * * column 4, line 58 - column 5, line 6 * * figure 1 * ---	1	A61B10/00 B01L3/14 B01L3/00
A	US 6 299 842 B1 (KOZAK KENNETH JAMES ET AL) 9 October 2001 (2001-10-09) * column 3, line 53 - column 4, line 13 * * figure 1 * -----	1	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			A61B B01L G01N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 21 July 2003	Examiner Compos, F
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 03 01 1896

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
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21-07-2003

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EP 0727653	A	21-08-1996	DE 69610569 D1	16-11-2000
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	15/634,607	Filing Date	2017-06-27	Docket Number (if applicable)	EXCTD-35239.304	Art Unit	1634
First Named Inventor	JOOST LOUWAGIE			Examiner Name	WHISENANT		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

☐ Other _____

☒ Enclosed

☐ Amendment/Reply

☒ Information Disclosure Statement (IDS)

☐ Affidavit(s)/ Declaration(s)

☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

☒ The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to
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Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2023-06-28
Name	Mary Ann D. Brow	Registration Number	42363

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

173254 7590 08/07/2023
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

EXAMINER

WHISENANT, ETHAN C

ART UNIT

PAPER NUMBER

1634

DATE MAILED: 08/07/2023

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874

TITLE OF INVENTION: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1200	\$0.00	\$0.00	\$1200	11/07/2023

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL **Patent Owner's Ex. 2003, Page 1338**

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
 Commissioner for Patents
 P.O. Box 1450
 Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

173254 7590 08/07/2023
 CASIMIR JONES, S.C.
 2275 DEMING WAY, SUITE 310
 MIDDLETON, WI 53562

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874

TITLE OF INVENTION: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1200	\$0.00	\$0.00	\$1200	11/07/2023

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHISENANT, ETHAN C	1634	435-006110

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

1 _____

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required)

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

☐ Electronic Payment via Patent Center or EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. Change in Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874
173254	7590	08/07/2023	EXAMINER	
CASIMIR JONES, S.C. 2275 DEMING WAY, SUITE 310 MIDDLETON, WI 53562			WHISENANT, ETHAN C	
			ART UNIT	PAPER NUMBER
			1634	
DATE MAILED: 08/07/2023				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.** Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Ethan C Whisenant	Art Unit 1634	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to 28 JUN 2023.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are See Continuation Sheet. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov**.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some* c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. ____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: ____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date <u>28 JUN</u> . 3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____. 4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____.	5. ☐ Examiner's Amendment/Comment 6. ☒ Examiner's Statement of Reasons for Allowance 7. ☐ Other _____.
---	--

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634

Continuation of 3. The allowed claim(s) is/are: 82,84,87,89-94 and 96-100

2023NOTICE OF ALLOWABILITY

► A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed on 28 JUN 2023 in this application after final action (i.e. an allowance). Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. **Claim(s) 82, 84, 87, 89-94 and 96-100** is/are now pending.

► The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

REASON(S) FOR ALLOWANCE

► **Claim(s) 82, 84, 87, 89-94 and 96-100** is/are deemed to be allowable for the reason(s) of record, see the NOA mailed 02 DEC 2022. The Claims have now been renumbered as Claims 1-14 in the same order as presented by the applicant.

Conclusion

C. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ethan Whisenant whose telephone number is (571) 272-0754. The examiner can normally be reached Monday-Friday from 8:30 am -5:30 pm EST or any time via voice mail. If repeated attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Wu-Cheng Winston Shen, can be reached at (571) 272-3157.

The Central Fax number for the USPTO is (571) 273-8300. Please note that the faxing of papers must conform with the Notice to Comply published in the Official Gazette, 1096 OG 30 (November 15, 1989).

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634
ethan.whisenant@uspto.gov

Search Notes 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

CPC - Searched*		
Symbol	Date	Examiner
C12Q 16886	11/22/2022	ECW
G01N 33/574	11/22/2022	ECW
Updated Search	03/24/2023	ECW
Updated Search	07/28/2023	ECW

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	
--	--

Search Notes 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

Search Notes		
Search Notes	Date	Examiner
EAST (see transcript for databases and search terms)	06/23/2019	B. L. S.
Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.
EAST (see transcript for databases and search terms)	01/14, 15/2020	B. L. S.
STN (see transcript for databases and search terms)	01/14/2020	B. L. S.
EAST (updated)	08/04/2020	B. L. S.
EAST	06/16/2021	B. L. S.
EAST	03/22/2022	B. L. S.
PE2E Search	06/09/2022	B. L. S.
See the Examiner's Search Notes on the last pg of the NOA	11/22/2022	ECW
Updated Search	03/24/2023	ECW
Updated Search	07/28/2023	ECW

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
C12Q	1/6886	11/22/2022	ECW
G01N	33/574	11/22/2022	ECW
Updated	Search	03/24/2023	ECW
Updated	Search	07/28/2023	ECW

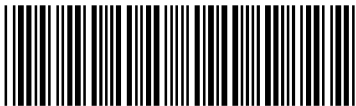
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	
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Issue Classification 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

CPC						
Symbol					Type	Version
C12Q	/	1	/	6886	F	2013-01-01
G01N	/	33	/	57446	I	2013-01-01
C12Q	/	2600	/	106	A	2013-01-01
C12Q	/	2600	/	154	A	2013-01-01
C12Q	/	2600	/	158	A	2013-01-01
G01N	/	2800	/	50	A	2013-01-01
G01N	/	2800	/	52	A	2013-01-01
G01N	/	2333	/	805	A	2013-01-01

CPC Combination Sets					
Symbol				Type	Set

NONE (Assistant Examiner) (Date)		Total Claims Allowed: 14	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Priamry Examiner) (Date)		O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost	
	Examiner Ethan C Whisenant	Art Unit 1634	

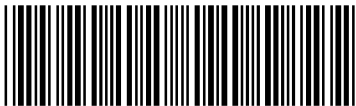
INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12Q1/6886	/	1	/ 6886
G01N33/574	/	33	/ 574

NON-CLAIMED			
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US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE (Assistant Examiner) (Date)		Total Claims Allowed: 14	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner) (Date)		O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

☒ Claims renumbered in the same order as presented by applicant
 ☐ CPA
 ☐ T.D.
 ☐ R.1.47

CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
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	4		17		30		43		56		69	1	82		95
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	12		25		38		51		64		77	5	90		
	13		26		39		52		65		78	6	91		

NONE (Assistant Examiner) _____ (Date) _____ /ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner) _____ (Date) _____	Total Claims Allowed: 14	
	O.G. Print Claim(s) 1	O.G. Print Figure None

<i>Index of Claims</i> 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS										
☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47										
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<i>Index of Claims</i> 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

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<i>Index of Claims</i> 	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

CLAIM		DATE								
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14	100	=	=							

Patent Owner's Ex. 2003, Page 1353

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT
Sheet	1	of	2	Attorney Docket Number	EXCTD-35239.304

U.S. PATENTS					
Exami ner Initials*	Cite No. ¹	Document Number Number-Kind Code ² (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		4859610	1989-08-22	MAGGIO	
		5137806	1992-08-11	LEMAISTRE et al.	
		5196167	1993-03-23	GUADAGNO et al.	
		5527676	1996-06-18	VOGELSTEIN et al.	
		5741650	1998-04-21	LAPIDUS et al.	
		5952178	1999-09-14	LAPIDUS et al.	
		11634781	2023-04-25	LOUWAGIE	

U.S. PUBLISHED PATENT APPLICATIONS					
Exami ner Initials*	Cite No. ³	Document Number Number-Kind Code ⁴ (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20070017015	2007-01-25	FINELL	
		20080097238	2008-04-24	LOKTIONOV et al.	

Note: Submission of copies of U.S. Patents and published U.S. Patent Applications is not required.

FOREIGN PATENT DOCUMENTS						
Exami ner Initials*	Cite No. ¹	Foreign Patent Document Country Code ⁵ Number ⁶ Kind Code ⁷ (if known)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translati on ⁸
		EP 1366715	2003-12-03	SENTINEL CH. S.r.L		
		WO 2005/014154	2005-02-17	LOOTS LIMITED		

NONPATENT LITERATURE DOCUMENTS			
Examin er Initials*	Cite No. ¹	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and-or country where published.	Translati on ⁶
Examiner Signature			Date Considered

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Patent Owner's Ex. 2003, Page 1354

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT
Sheet	2	of	2	Attorney Docket Number	EXCTD-35239.304

NONPATENT LITERATURE DOCUMENTS			
		AHLQUIST et al., Stool DNA and Occult Blood Testing for Screen Detection of Colorectal Neoplasia. Ann Intern Med. 2008 Oct 7;149(7):441-W81	
		HOEPFFNER et al., Comparative evaluation of a new bedside faecal occult blood test in a prospective multicentre study. Aliment Pharmacol Ther. 2006 Jan 1;23(1):145-54	
		LEUNG et al., Detection of Hypermethylated DNA or Cyclooxygenase-2 Messenger RNA in Fecal Samples of Patients with Colorectal Cancer or Polyps. Am J Gastroenterol 2007;102:1070-6	
		OHLSSON et al., Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR. Br J Cancer. 2006 Jul 17;95(2):218-25	
		OLSON et al., DNA Stabilization is Critical for Maximizing Performance of Fecal DNA-Based Colorectal Cancer Tests. Diagn Mol Pathol 2005;14:183-191	
		Request for Ex Parte Reexamination of U.S. Patent 11,634,781, filed May 22, 2023, 286 pages	
		Declaration of Mr. Anthony P. Shuber, MS, Reexam Application 90/015,237, filed May 22, 2023, 63 pages	

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	07/15/2023
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EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Patent Owner's Ex. 2003, Page 1355

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				<i>Application Number</i>	15/634,607
				<i>Filing Date</i>	06/27/2017
				<i>First Named Inventor</i>	JOOST LOUWAGIE
				<i>Art Unit</i>	1634
				<i>Examiner Name</i>	WHISENANT
Sheet	3	of	2	<i>Attorney Docket Number</i>	EXCTD-35239.304

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17(p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

☐ Documents not provided herewith have been previously cited in parent U.S. patent application number _____ filed on _____. In compliance with 37 C.F.R. § 1.98(d), Applicants have not included copies of these documents.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2023-06-28
Name/Print	Mary Ann D. Brow	Registration Number	42363

Patent Owner's Ex. 2003, Page 1356

PTO Notes regarding this form:

¹ Applicant's unique citation designation number (optional).

² See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04.

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⁷ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible.

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This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 173254

Mon Aug 07 05:05:58 EDT 2023

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Application	Document	Mailroom Date	Attorney Docket No.
15634607	NOA	08/07/2023	EXCTD-35239.304
15634607	1449	08/07/2023	EXCTD-35239.304

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Sincerely,

The Patent Center Team

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #
15/634,607

RECEIPT DATE / TIME
11/06/2023 10:34:56 PM Z ET

ATTORNEY DOCKET #
EXCTD-35239.304

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE Utility - Nonprovisional Application
under 35 USC 111(a)

PATENT # -

CONFIRMATION # 1874

FILED BY Mary Ann Brow

PATENT CENTER # 63175645

FILING DATE 06/27/2017

CUSTOMER # 173254

FIRST NAMED INVENTOR J oost Louwagie

CORRESPONDENCE ADDRESS -

AUTHORIZED BY -

Documents

TOTAL DOCUMENTS: 3

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-11-06_Rule312_Amendment_EXCTD-003USCON2-35239-304.pdf	5	-	157 KB
2023-11-06_Rule312_Amendment_EXCTD-003USCON2-35239-304-A.NA.pdf	(1-1) 1	Amendment after Notice of Allowance (Rule 312)	136 KB
2023-11-06_Rule312_Amendment_EXCTD-003USCON2-35239-304-CLM.pdf	(2-4) 3	Claims	141 KB

2023-11-06_Rule312_Amendment_EXC XCTD-003USCON2-35239-304-REM.pdf	(5-5)	1	Applicant Arguments/Remarks Made in an Amendment	136 KB
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Digest

DOCUMENT

MESSAGE DIGEST(SHA-512)

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Joost Louwagie	Confirmation: 1874
Serial No.: 15/634,607	Group No.: 1634
Filed: 06/27/2017	Examiner: Ethan C. Whisenant
Entitled: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

**AMENDMENT AFTER ALLOWANCE
UNDER 37 C.F.R. §1.312**

EFS WEB FILED
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This communication is a request to enter an amendment to the claims after receipt of a Notice of Allowance under 37 C.F.R. §1.312. Applicants respectfully request entry of this amendment.

Amendments to the Claims are reflected in the Listing of Claims beginning on Page 2;
Remarks are on Page 5.

AMENDMENTS TO THE CLAIMS:

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

- 1-81. (canceled)
82. (previously presented) A method of processing a freshly-collected fecal sample without freezing, the method comprising:
- a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject by defecation directly into a sealable collection vessel;
 - b) removing a portion of said fecal sample to a separate container to produce a removed portion and a remaining portion of said fecal sample;
 - c) combining the removed portion in the separate container with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample; and
 - d) stabilizing the remaining portion of the fecal sample by adding a buffer that maintains DNA integrity in a fecal sample to the remaining portion in the sealable collection vessel.
83. (canceled)
84. (previously presented) The method of claim 82, further comprising delivering the container containing the removed portion and buffer and the sealable vessel containing the remaining portion of the fecal sample and buffer to a medical diagnostics laboratory.
- 85-86. (canceled)
87. (currently amended) A method of processing a fecal sample, the method comprising:
- A) obtaining a pair of portions of a fecal sample from a human subject, the pair of

portions comprising a container containing a removed portion and a buffer, and a sealable vessel containing a stabilized remaining portion of a fecal sample and a buffer obtained by the method of claim 82[[],];

- B) testing the removed portion of said fecal sample for a concentration of hemoglobin present in said removed portion[[],];
- C) extracting DNA from the remaining portion of said fecal sample; and
- D) analyzing said DNA for the presence of DNA from the human subject having an epigenetic modification.

88. (canceled)

89. (previously presented) The method of claim 87, wherein testing for the concentration of hemoglobin in said removed portion comprises immunochemical detection of hemoglobin.

90. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml.

91. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml.

92. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml.

93. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 50 ng/ml.

94. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml.
95. (canceled)
96. (previously presented) The method of claim 87, wherein said epigenetic modification comprises aberrant methylation.
97. (currently amended) The method of claim ~~87~~96, wherein said aberrant methylation comprises hypermethylation.
98. (previously presented) The method of claim 87, wherein said DNA from the human subject comprises a gene and/or a promoter region of a gene.
99. (previously presented) The method of claim 98, wherein said gene is selected from the group consisting of PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2, and CDO1.
100. (previously presented) The method of claim 96, wherein analyzing said DNA comprises modifying said DNA with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.

REMARKS

Claim 87 is herein amended to replace commas with semicolons at the end of part A) and part B), for consistency with part C) of claim 87.

Claim 97 is herein amended to depend from claim **96** instead of from claim **87**.

These amendments are to correct typographical errors, and do not add new matter or change the scope of the claims.

Applicant respectfully requests entry of this amendment.

CONCLUSION

Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicant encourages the Examiner to call the undersigned at (608) 662-1277.

Dated: November 6, 2023

/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

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #
15/634,607

RECEIPT DATE / TIME
11/07/2023 02:56:25 PM Z ET

ATTORNEY DOCKET #
EXCTD-35239.304

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under 35 USC 111(a)

PATENT # -

CONFIRMATION # 1874

FILED BY Stephanie Filandrinos

PATENT CENTER # 63181152

FILING DATE 06/27/2017

CUSTOMER # 173254

FIRST NAMED INVENTOR J oost Louwagie

CORRESPONDENCE ADDRESS -

AUTHORIZED BY Mary Ann Brow

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-11-07_35239.304_CommentsOn Allowance.pdf	2	Post Allowance Communication - Incoming	200 KB

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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Joost Louwagie	Confirmation: 1874
Serial No.: 15/634,607	Group No.: 1634
Filed: 06/27/2017	Examiner: Ethan C. Whisenant
Entitled: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

**COMMENTS REGARDING EXAMINER'S STATEMENT ON
REASONS FOR ALLOWANCE MAILED 07 AUG 2023**

EFS WEB FILED
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Applicant is filing the following comments in response to the Examiner's statement of Reasons for Allowance included with the Notice of Allowance mailed 07 AUG 2023 ("Notice of Allowance") for the above-referenced application. Applicant agrees with the Examiner's conclusion that claims 82, 84, 87, 89-94 and 96-100 are allowable for reasons that are clear from the record of the prosecution as a whole.

Applicant notes that page 2 of the Notice of Allowance includes the text reproduced below:

REASON(S) FOR ALLOWANCE

► **Claim(s) 82, 84, 87, 89-94 and 96-100** is/are deemed to be allowable for the reason(s) of record, see the NOA mailed 02 DEC 2022. The Claims have now been renumbered as Claims 1-14 in the same order as presented by the applicant.

Applicant notes that the Reasons for Allowance stated in the NOA mailed on 02 DEC 2022 states following:

REASON(S) FOR ALLOWANCE

► **Claim(s) 82, 84, 87, 89-94 and 96-100** is/are deemed to be allowable in light of the paper(s) filed 10 AUG 2022 and the persuasive argument(s) therein. The Claims have now been renumbered as Claims 1-14 in the same order as presented by the applicant.

Applicant notes that no paper or arguments were filed by the Applicant on 10 AUG 2022. Applicant filed a Notice of Appeal with a Pre-Appeal Brief Review (“PABR”) request with an accompanying brief on 27 JUNE 2022. On the basis of arguments made in that brief, Applicant received a Notice of Panel Decision re-opening prosecution on 10 AUG 2022.

It is Applicant’s position that the Reasons for Allowance included in the Examiner’s statement of Reasons for Allowability mailed 02 DEC 2022, and now cited by the Examiner in the Notice of Allowance mailed 07 AUG 2023, acknowledges the persuasive arguments filed by Applicant in the PABR brief filed 27 JUNE 2022, as acknowledged by the Panel in the Notice of Panel Decision mailed 10 AUG 2022.

It is further Applicant’s position that the Examiner’s statement as a whole, including the dates on which arguments were stated as having been filed, has no bearing on the allowability, scope, interpretation, or any other aspect of any of allowed claims 1-14 of the instant application. Applicant expressly reserves the right to set forth additional and/or alternative reasons for the patentability of the claims in future applications and/or proceedings.

Dated: November 7, 2023

/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

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #
15/634,607

RECEIPT DATE / TIME
11/07/2023 03:05:06 PM Z ET

ATTORNEY DOCKET #
EXCTD-35239.304

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE Utility - Nonprovisional Application
 under 35 USC 111(a)

PATENT # -

CONFIRMATION # 1874

FILED BY Stephanie Filandrinos

PATENT CENTER # 63183007

FILING DATE 06/27/2017

CUSTOMER # 173254

FIRST NAMED
INVENTOR J oost Louwagie

CORRESPONDENCE
ADDRESS -

AUTHORIZED BY Mary Ann Brow

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-11-07_35239.304_IssueFeeTransmittal.pdf	2	Issue Fee Payment (PTO-85B)	263 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2023-11-07_35239.304_IssueFeeTransmittal.pdf	71216E6AEF42C11B79C557E6997D5D9716F134CE069FF9A7F DCD4D932D6F1E777E87B32161F0C3CF26A24A4EAE4A9BD6B 55397A8BD53545AAD943C227D8EA3F1

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Patent Owner's Ex. 2003, Page 1332 B – FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
 Commissioner for Patents
 P.O. Box 1450
 Alexandria, VA 22313-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

173254
 Casimir Jones, S.C.
 2275 Deming Way, Suite 310
 Middleton, WI 53562

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

Stephanie Filandrinos	(Typed or printed name)
/Stephanie Filandrinos/	(Signature)
11/07/2023	(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
15/634,607	06/27/2017	LOUWAGIE, Joost	EXCTD-35239.304	1874

TITLE OF INVENTION:

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
CON	LE	\$1200	\$0.00	\$0.00	\$1200	11/07/2023

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHISENANT, ETHAN	1634	435/006110

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363)

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. Casimir Jones, S.C.
 2. Mary Ann D. Brow
 3.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

Exact Sciences Corporation

(B) RESIDENCE: (CITY and STATE or COUNTRY)

Madison, WI

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government4a. Fees Submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment (Please first reapply any previously paid fee shown above):

- ☒ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)
- ☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 504302

5. Change of Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29.
- ☐ Applicant asserting small entity status. See 37 CFR 1.27.
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Mary Ann D. Brow/

Date 11/07/2023

Typed or printed name Mary Ann D. Brow

Registration No. 42,363

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.** Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ELECTRONIC PAYMENT RECEIPT

APPLICATION #
15/634,607

RECEIPT DATE / TIME
11/07/2023 03:05:06 PM Z ET

ATTORNEY DOCKET #
EXCTD-35239.304

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE Utility - Nonprovisional Application
under 35 USC 111(a)

PATENT # -

CONFIRMATION # 1874

FILED BY Stephanie Filandrinos

PATENT CENTER # 63183007

AUTHORIZED BY Mary Ann Brow

CUSTOMER # 173254

FILING DATE 06/27/2017

CORRESPONDENCE ADDRESS -

FIRST NAMED INVENTOR Joost Louwagie

Payment Information

PAYMENT METHOD
CARD / 0638

PAYMENT TRANSACTION ID
E2023A7F05347601

PAYMENT AUTHORIZED BY
Stephanie Filandrinos

FEE CODE	DESCRIPTION	ITEM PRICE(\$)	QUANTITY	ITEM TOTAL(\$)
1501	UTILITY ISSUE FEE	1200.00	1	1200.00
TOTAL AMOUNT:				\$1,200.00

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

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submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Patent Owner's Ex. 2003, Page 13 **BRINTER RUSH**
(PTO ASSISTANCE)

Application: 15634607

Examiner: Whisenant

GAU: 1634

From:

Location: RTFM

Creation Date: 11/07/2023

Week Date: 11/28/2022

<u>DOC CODE</u>	<u>DOC DATE</u>	<u>MISCELLANEOUS</u>
☐ 1449		☐ Continuing Data
☐ IDS		☐ Foreign Priority
☐ CLM		☐ Document Legibility
☐ IIFW/FWCLM		☐ Fees
☐ SRFW		☐ Petition (TC)
☐ DRW		☐ Other
☐ OATH		
☒ 312	<u>11/06/2023</u>	
☐ SPEC		

[RUSH] Message:

Please respond to the 11/6/23 a.na

thank you

[XRUSH] Response:

Initials: _____



UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874
173254	7590	11/15/2023	EXAMINER	
CASIMIR JONES, S.C. 2275 DEMING WAY, SUITE 310 MIDDLETON, WI 53562			WHISENANT, ETHAN C	
			ART UNIT	PAPER NUMBER
			1634	
			NOTIFICATION DATE	DELIVERY MODE
			11/15/2023	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DOCKETING@CASIMIRJONES.COM
Genomic_docketing@cardinal-ip.com

RESPONSE TO 312 AMENDMENT

1. The applicant's amendment filed 07 NOV 2023 under 37 CFR 1.312 has been entered.

Conclusion

C. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ethan Whisenant whose telephone number is (571) 272-0754. The examiner can normally be reached Monday-Friday from 8:30 am -5:30 pm EST or any time via voice mail. If repeated attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Wu-Cheng Winston Shen, can be reached at (571) 272-3157.

The Central Fax number for the USPTO is (571) 273-8300. Please note that the faxing of papers must conform with the Notice to Comply published in the Official Gazette, 1096 OG 30 (November 15, 1989).

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634
ethan.whisenant@uspto.gov

Response to Rule 312 Communication	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Ethan C Whisenant	Art Unit 1634	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☒ The amendment filed on 07 November 2023 under 37 CFR 1.312 has been considered, and has been:
- a) ☐ entered.
 - b) ☒ entered as directed to matters of form not affecting the scope of the invention.
 - c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.
 - d) ☐ disapproved. See explanation below.
 - e) ☐ entered in part. See explanation below.
 - f) ☐ not entered because the supplemental or corrected Application Data sheet (ADS)
 - ☐ was not accompanied by a petition to accept an unintentionally delayed claim under 37 CFR 1.55 or 27 CFR 1.78;
 - ☐ did not identify the information being changed in accordance with 37 CFR 1.76(c)(2);
 - ☐ was not properly signed in accordance with 37 CFR 1.76(e) (or 37 CFR 1.33(b) for applications filed prior to September 16, 2012).

See the Interview Summary

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634

<i>Applicant-Initiated Interview Summary</i>	Application No. 15/634,607	Applicant(s) Louwagie, Joost		
	Examiner Ethan C Whisenant	Art Unit 1634	AIA (First Inventor to File) Status No	Page 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	Title	Type
Ethan C Whisenant	Primary Examiner	Telephonic
Mary Ann Brow	Attorney of Record	

Date of Interview: 02 November 2023

Issues Discussed:

Proposed Amendment(s)

The applicant inquired about a potential 312 amendment. The Examiner indicated that the amendment, as presented, would be entered.

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	
Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04 Please further see: MPEP 713.04 Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b) 37 CFR § 1.2 Business to be transacted in writing	

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	1874
Serial No.:	15/634,607	Group No.:	1634
Filed:	06/27/2017	Examiner:	Ethan C. Whisenant
Entitled:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD		

**AMENDMENT AFTER ALLOWANCE
UNDER 37 C.F.R. §1.312**

EFS WEB FILED
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This communication is a request to enter an amendment to the claims after receipt of a Notice of Allowance under 37 C.F.R. §1.312. Applicants respectfully request entry of this amendment.

Amendments to the Claims are reflected in the Listing of Claims beginning on Page 2;

Remarks are on Page 5.

AMENDMENTS TO THE CLAIMS:

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

1-81. (canceled)

82. (previously presented) A method of processing a freshly-collected fecal sample without freezing, the method comprising:

- a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject by defecation directly into a sealable collection vessel;
- b) removing a portion of said fecal sample to a separate container to produce a removed portion and a remaining portion of said fecal sample;
- c) combining the removed portion in the separate container with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample; and
- d) stabilizing the remaining portion of the fecal sample by adding a buffer that maintains DNA integrity in a fecal sample to the remaining portion in the sealable collection vessel.

83. (canceled)

84. (previously presented) The method of claim 82, further comprising delivering the container containing the removed portion and buffer and the sealable vessel containing the remaining portion of the fecal sample and buffer to a medical diagnostics laboratory.

85-86. (canceled)

87. (currently amended) A method of processing a fecal sample, the method comprising:

- A) obtaining a pair of portions of a fecal sample from a human subject, the pair of

portions comprising a container containing a removed portion and a buffer, and a sealable vessel containing a stabilized remaining portion of a fecal sample and a buffer obtained by the method of claim 82[[],];

- B) testing the removed portion of said fecal sample for a concentration of hemoglobin present in said removed portion[[],];
- C) extracting DNA from the remaining portion of said fecal sample; and
- D) analyzing said DNA for the presence of DNA from the human subject having an epigenetic modification.

88. (canceled)

89. (previously presented) The method of claim 87, wherein testing for the concentration of hemoglobin in said removed portion comprises immunochemical detection of hemoglobin.

90. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml.

91. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml.

92. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml.

93. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 50 ng/ml.

94. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml.
95. (canceled)
96. (previously presented) The method of claim 87, wherein said epigenetic modification comprises aberrant methylation.
97. (currently amended) The method of claim ~~87~~96, wherein said aberrant methylation comprises hypermethylation.
98. (previously presented) The method of claim 87, wherein said DNA from the human subject comprises a gene and/or a promoter region of a gene.
99. (previously presented) The method of claim 98, wherein said gene is selected from the group consisting of PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2, and CDO1.
100. (previously presented) The method of claim 96, wherein analyzing said DNA comprises modifying said DNA with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.

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SUBJECT: USPTO: Patent Electronic System - Correspondence Notification for Customer Number 173254

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Application	Document	Mailroom Date	Attorney Docket No.
15634607	N271	11/15/2023	EXCTD-35239.304
15634607	EXIN	11/15/2023	EXCTD-35239.304

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	12/19/2023	11845991	EXCTD-35239.304	1874

173254 7590 11/29/2023
CASIMIR JONES, S.C.
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MIDDLETON, WI 53562

ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at <https://patentcenter.uspto.gov/>. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

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Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 903 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

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INVENTOR(s) (Please see PATENT CENTER site <https://patentcenter.uspto.gov> for additional inventors):

Joost Louwagie, Dornach, SWITZERLAND;

APPLICANT(s) (Please see PATENT CENTER site <https://patentcenter.uspto.gov> for additional applicants):

Exact Sciences Corporation, Madison, WI;

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Application	Document	Mailroom Date	Attorney Docket No.
15634607	ISSUE.NTF	11/29/2023	EXCTD-35239.304

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Joost Louwagie	Confirmation: 1874
Serial No.: 15/634,607	Group No.: 1634
Filed: 06/27/2017	Examiner: Ethan C. Whisenant
Entitled: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

APPLICANT'S INTERVIEW SUMMARY

EFS WEB FILED
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Examiner Whisenant

This communication is responsive to the communication issued November 15, 2023.
Applicant's counsel thanks the Examiner for the interview of November 2, 2023, where potential
312 amendments were discussed.

Dated: December 6, 2023

/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



ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #
15/634,607

RECEIPT DATE / TIME
12/06/2023 03:15:58 PM Z ET

ATTORNEY DOCKET #
EXCTD-35239.304

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE Utility - Nonprovisional Application
under 35 USC 111(a)

PATENT # 11845991

CONFIRMATION # 1874

FILED BY Stephanie Filandrinos

PATENT CENTER # 63507147

FILING DATE 06/27/2017

CUSTOMER # 173254

FIRST NAMED
INVENTOR Joost Louwagie

CORRESPONDENCE
ADDRESS -

AUTHORIZED BY Mary Ann Brow

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-12-06_35239.304_InterviewSummary.pdf	1	Applicant summary of interview with examiner	129 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2023-12-06_35239.304_InterviewSummary.pdf	42E73B80E42249C4ACC312A9BFD1B9E94423114E5B4CEE5C BEAD9CF48CEECE5CC335CD4CC452207F6BB8416CD0590717 2C71841B931B85B35408C5567A6A4EAF

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874
173254	7590	12/19/2023	EXAMINER	
CASIMIR JONES, S.C. 2275 DEMING WAY, SUITE 310 MIDDLETON, WI 53562			WHISENANT, ETHAN C	
			ART UNIT	PAPER NUMBER
			1634	
			NOTIFICATION DATE	DELIVERY MODE
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APPLICATION NO.	ISSUE DATE	PATENT NO.
15/634,607	19-Dec-2023	11845991

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