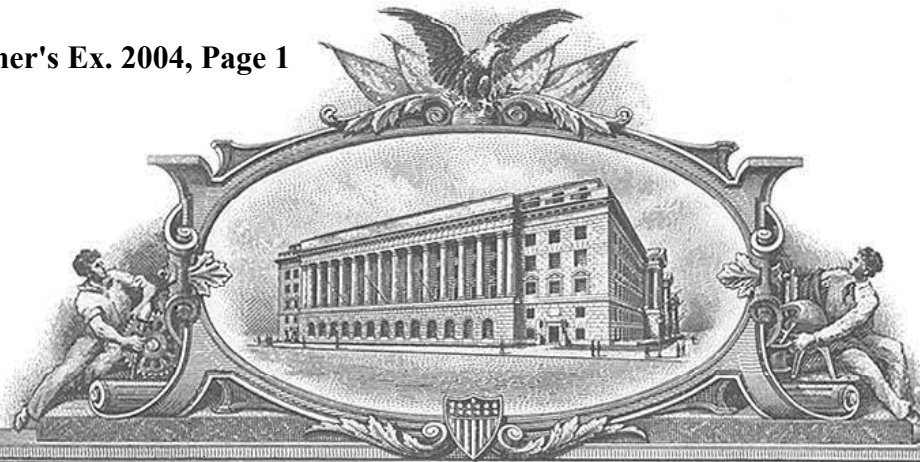


8442974



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

December 21, 2023

**THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE
RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS
OF:**

**APPLICATION NUMBER: 17/936,335
FILING DATE: *September 28, 2022*
PATENT NUMBER: 11634781
ISSUE DATE: *April 25, 2023***



Certified by

Kathi

Performing the Functions and Duties of the
Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Joost Louwagie	Nonprovisional Application Number (if known):	
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently \$0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.
2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.
3. The applicable box is checked below:
 - I. ☒ **Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)**
 - i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.
---OR---
 - (b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.
 - ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, or the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.
 - II. ☐ **Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)**
 - i. A request for continued examination has been filed with, or prior to, this form.
 - ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
 - iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
 - iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
 - v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Mary Ann D. Brow/	Date 2022-09-28
Name (Print/Typed) Mary Ann D. Brow	Practitioner Registration Number 42363

Note: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.*

☒ *Total of 1 forms are submitted.

Privacy Act Statement

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

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	1	of	5	Attorney Docket Number	EXCTD-35239.306

U.S. PATENTS					
Exami ner Initials*	Cite No. ¹	Document Number	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
		Number-Kind Code ² <i>(if known)</i>			
		4437975	1984-03-20	GILLESPIE et al.	
		4445235	1984-05-01	SLOVER et al.	
		4582811	1986-04-15	PUCCI et al.	
		4683197	1987-07-28	GALLATI	
		4889818	1989-12-26	GELFAND et al.	
		5198365	1993-03-30	GROW et al.	
		6410276	2002-06-25	BURG et al.	
		7288413	2007-10-30	GOULDEN	
		7371527	2008-05-13	BAYLIN et al.	
		8969046	2015-05-03	VAN ENGELAND et al.	

U.S. PUBLISHED PATENT APPLICATIONS					
Exami ner Initials*	Cite No. ³	Document Number	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
		Number-Kind Code ⁴ <i>(if known)</i>			
		20020187476	2002-12-12	KOROULIS et al.	
		20030086869	2003-05-08	STALLINGS	
		20030096244	2003-05-22	RABELLO et al.	
		20040019298	2004-01-29	ZHOU et al.	
		20040091881	2004-05-13	OLEK et al.	
		20050064401	2005-03-24	OLEK et al.	
		20050075543	2005-04-07	CALABRESE	
		20060084054	2006-04-20	ALSOBROOK et al.	
		20060188939	2006-08-24	GAO	
		20060210448	2006-09-21	WANG et al.	
		20060216830	2006-09-28	KIKUIRI	
		20070017015	2007-01-25	FINELL	
		20080097238	2008-04-24	LOKTIONOV et al.	

Examiner Signature		Date Considered	
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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.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	2	of	5	Attorney Docket Number	EXCTD-35239.306

U.S. PUBLISHED PATENT APPLICATIONS					
		20080124714	2008-05-29	SHUBER et al.	
		20080221056	2008-09-11	BAYLIN et al.	
		20080227208	2008-09-18	YEE et al.	
		20090004058	2009-01-01	LIANG et al.	
		20100136572	2010-06-03	ATAMAN-ONAL et al.	
		20120164238	2012-06-28	JOOST	
		20160194723	2016-07-07	JOOST	

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	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
		Country Code ⁵ Number ⁶ Kind Code ⁷ <i>(if known)</i>				
		EP 0032782	1981-07-29	KOMMANDIITTIYHTIO FINNPIPETTE		
		EP 0308227	1989-03-22	SMITHKLINE DIAGNOSTICS, INC.		
		EP 0817968	1998-01-14	EXACT SCIENCES CORPORATION		
		FR 2919065	2009-01-23	BIOMERIEUX		
		WO 1990/06995	1990-06-28	SISKA DIAGNOSITCS, INC.		
		WO 1997/46705	1997-12-11	THE JOHNS HOPKINS SCHOOL OF MEDICINE		
		WO 2004/067726	2004-08-12	KECK GRADUATE INSTITUTE		
		WO 2004/083399				
		WO 2004/0927209	2004-10-28	KIETURAKIS & KARCZEWSKI		
		WO 2005/017207	2005-02-04	CASE WESTERN RESERVE UNIVERSITY		
		WO 2006/113671	2006-10-26	ONCOMETHYLOME SCIENCES SA		

Examiner Signature		Date Considered	
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	3	of	5	Attorney Docket Number	EXCTD-35239.306

FOREIGN PATENT DOCUMENTS						
		WO 2008/010975	2008-01-24	THE JOHNS HOPKINS UNIVERSITY		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		
		WO 2008/100913	2008-08-21	THE JOHNS HOPKINS UNIVERSITY		
		WO 2008/102002	2008-08-28	RIKSHOSPITALET RADIUMHOSPITALE		

NONPATENT LITERATURE DOCUMENTS				
Examiner 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.		Translation ⁶
		ABBASZADEGAN et al., Stool-based DNA testing, a new noninvasive method for colorectal cancer screening, the first report from Iran. World J Gastroenterol. 2007 Mar 14;13(10):1528-33		
		AHLQUIST et al., Cologuard Primed to Change Landscape of CRC Screenin", Mayo Clinic Clinical Updates, http://www.mayoclinic.org/medical-professionals/clinical-updates/digestivediseases/cologuard-primed-to-change-landscape-of-crc-screening , Pages 1-4, 12/03/2014.		
		AUERKARI, Methylation of tumor suppressor genes p16(INK4a), p27(Kip1) and E-cadherin in carcinogenesis. Oral Oncol. 2006 Jan;42(1):5-13		
		CHEN et al., Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst. 2005 Aug 3;97(15):1124-32		
		CONROY et al., Exact Sciences Completes 40,000 Cologuard Tests During First Quarter 2016, Exact Sciences Latest News, Pages 1-10, 05/03/2016.		
		EXACT SCIENCES, Cologuard(TM) sDNA-based Colorectal Cancer Screening Test - Instructions for Use, Pages 1-71, 2013.		
		HAMMER, Human hybrids. Sci Am. 2013 May;308(5):66-71		
		ITZKOWITZ et al., A simplified, noninvasive stool DNA test for colorectal cancer detection. Am J Gastroenterol. 2008 Nov;103(11):2862-70		
		KARL et al., Improved diagnosis of colorectal cancer using a combination of fecal occult blood and novel fecal protein markers. Clin Gastroenterol Hepatol. 2008 Oct;6(10):1122-8		
		KIM et al., Noninvasive Molecular Biomarkers for the Detection of Colorectal Cancer. BMB Rep. 2008 Oct 31;41(10):685-92		

Examiner Signature		Date Considered	
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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.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	4	of	5	Attorney Docket Number	EXCTD-35239.306

NONPATENT LITERATURE DOCUMENTS					
		KUTZNER et al., Non-invasive detection of colorectal tumours by the combined application of molecular diagnosis and the faecal occult blood test. Cancer Lett. 2005 Nov 8;229(1):33-41			
		LAIRD, The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003 Apr;3(4):253-66			
		LENHARD et al., Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. Clin Gastroenterol Hepatol. 2005 Feb;3(2):142-9			
		LEVI et al., A quantitative immunochemical faecal occult blood test is more efficient for detecting significant colorectal neoplasia than a sensitive guaiac test. Aliment Pharmacol Ther. 2006 May 1;23(9):1359-64			
		MULDER et al., Tumor pyruvate kinase isoenzyme type M2 and immunochemical fecal occult blood test: performance in screening for colorectal cancer. Eur J Gastroenterol Hepatol. 2007 Oct;19(10):878-82			
		REIN et al., Identifying 5-methylcytosine and related modifications in DNA genomes. Nucleic Acids Res. 1998 May 15;26(10):2255-64			
		SHASTRI et al., Comparison of an established simple office-based immunological FOBT with fecal tumor pyruvate kinase type M2 (M2-PK) for colorectal cancer screening: prospective multicenter study. Am J Gastroenterol. 2008 Jun;103(6):1496-504			
		SOMMER et al., Minimal homology requirements for PCR primers. Nucleic Acids Res. 1989 Aug 25;17(16):6749			
		TURGEON et al., Fecal DNA-Based Detection of Colorectal Neoplasia. Curr Colorectal Cancer Rep. 2007 Oct;3(4):171-177			
		VILLAR-GAREA et al., DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Curr Drug Metab. 2003 Feb;4(1):11-31			

Examiner Signature		Date Considered	
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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.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	5	of	5	Attorney Docket Number	EXCTD-35239.306

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 13/147,570 filed on March 12, 2012; 15/010,436, filed on January 29, 2016; and/or 15/634,607 filed on June 27, 2017. 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)	2022-09-28
Name/Print	Mary Ann D. Brow	Registration Number	42363

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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⁶ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document.

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

⁸ Applicant is to place a check mark here if English language Translation is attached.

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.**

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #
17/936,335

RECEIPT DATE / TIME
09/28/2022 07:52:03 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 #	1513	FILED BY	Stephanie Filandrinos
PATENT CENTER #	61015215	FILING DATE	-
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 8

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
generatedADS61015215.pdf	6	Application Data Sheet	135 KB
35239-306_SEQUENCE_LISTING.xml	-	Sequence Listing (ST.26 XML File)	35 KB
2022-09-28_35239-306_TrackOne_Form.pdf	2	Track One Request	112 KB
35239US4CON_Exec_Decl.pdf	1	Oath or Declaration filed	106 KB

Warning: The attached file contains comments or annotations. Comments are not recommended and may cause processing problems with the document.

Patent Owner's Ex. 2004, Page 11

2022-09-28_35239-306_IDS-SB08.pdf	6	Information Disclosure Statement (IDS) Form (SB08)	192 KB
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2022-09-28_35239-306_APPLICATION2.pdf	61	-	577 KB
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2022-09-28_35239-306_APPLICATION2-CLM.pdf	(57-60)	4	Claims	140 KB
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2022-09-28_35239-306_APPLICATION2-ABST.pdf	(61-61)	1	Abstract	107 KB
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327F30630D770B0AAF6C9B5F1AD07

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.

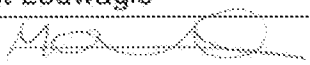
PTO/AIA/01 (08-12)

Approved for use through 01/31/2014. OMB 0651-0032

U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Methods of Detecting Colorectal Cancer
As the below named inventor, I hereby declare that: This declaration is directed to: ☐ The attached application, or ☒ United States application or PCT international application number <u>15/010,436</u> filed on <u>29-Jan-2016</u> The above-identified application was made or authorized to be made by me. I believe that I am the original inventor or an original joint inventor of a claimed invention in the application. I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both. WARNING: Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.	
LEGAL NAME OF INVENTOR	
Inventor: <u>Joost Louwagie</u> Date (Optional): <u>23 Jan 2016</u>	
Signature: 	
Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/US/AIA01 form for each additional inventor.	

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FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a continuation of U.S. Patent Application No. 15/634,607, filed
June 27, 2017, which is a continuation of U.S. Application No. 15/010,436, filed January 29,
2016, now abandoned, which is a continuation of U.S. Application No. 13/147,570, filed
March 12, 2012, now abandoned, which is the U.S. national stage of International
Application PCT/GB2010/000180, filed February 3, 2010, which claims priority to U.S.
10 Provisional Application No. 61/149,581, filed February 3, 2009. The contents of these
applications are incorporated herein by reference in their entireties.

SEQUENCE LISTING

 The text of the computer readable sequence listing filed herewith, titled “35239-
306_SEQUENCE_LISTING”, created September 28, 2022, having a file size of 35,630
15 bytes, is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

 The present invention relates to methods and kits for analysis of fecal samples
comprising removing a portion of a collected fecal sample and adding the removed portion of
20 the fecal sample to a buffer that prevents denaturation or degradation of blood proteins found
in the sample, and comprising detection of blood in the removed portion of the fecal sample,
e.g., by immunoassay.

BACKGROUND OF THE INVENTION

 Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide, and is
25 the second leading cause of cancer-related deaths in the United States. A patient's prognosis
is good if the cancer is caught early, when the site of the cancer is confined to its site of
origin. However, the cure rates fall once the cancer has spread. Most colon cancers arise from

conventional adenomatous polyps (conventional adenoma-to-carcinoma sequence), while some colon cancers appear to arise from the recently recognized serrated adenomatous polyp (serrated adenoma-to-carcinoma theory). Because conventional adenomas and serrated adenomas are usually asymptomatic, mass screening of asymptomatic patients has become the cornerstone for detecting and eliminating these precursor lesions to reduce the risk of colon cancer.

A number of different screening methods for CRC are available. Procedures such as digital rectal examination (DRE); colonoscopy or sigmoidoscopy are highly invasive, painful and can cause a great deal of patient discomfort. Other less invasive screening tests include fecal occult blood test (FOBT); fecal immunochemical test (FIT); barium enema with air contrast; virtual colonoscopy; biopsy (e.g., CT guided needle biopsy); and imaging techniques (e.g., ultrasound, CT scan, PET scan, and MRI).

Colonoscopy has become the primary screening test for CRC because of its high sensitivity and specificity, and the ability to perform polypectomy. While sensitive and specific, the procedure is invasive, costly, has limited availability and includes certain risks such as induction of infection and perforation of the bowel.

A commonly used and less expensive way of screening for CRC is a fecal occult blood test (FOBT), which tests for the presence of blood in faeces. The presence of haemoglobin as a representative blood protein in faeces is an indicator of intestinal bleeding, which is frequently associated with CRC. However, since occult in a fecal sample could be indicative of a variety of gastrointestinal disorders, further medical testing such as colonoscopy remains necessary to identify colorectal cancer.

Fecal occult blood tests fall primarily into two categories, tests based on the use of chromogenic chemical reagents such as gum guaiac and immunochemical tests. The chemically based guaiac methods determine the presence of occult blood by the detection of the peroxidase activity of the hemoglobin in the blood present in the faecal sample. They require catalysis of peroxide into oxygen and water, and the subsequent oxidation of a colorless dye (most often into a colored form). However, peroxidase activity is also found in

meats and vegetables. In order to produce accurate results, these tests require restriction of the intake of certain foods, drugs, vitamins, and other substances prior to and during the sample collection period. The sensitivity of the most commonly used guaiac FOBT (Hemoccult) is approximately 50%. Despite a specificity of 98%, the positive predictive value for FOBT is low. Methods of detecting occult blood based on porphyrin (heme and protoporphyrin IX) analysis or immunologic tests using anti-hemoglobin antibodies improve on these results. Immunochemical tests (FIT or iFOBT) that use anti-hemoglobin antibodies specific for human blood in extracts from stool do not require dietary restrictions; however, they are more complicated and more expensive than peroxidase-based tests. In addition, human hemoglobin in fecal samples degrades with time, resulting in a loss of antigenicity which can produce false negative results. Reported sensitivity of these immunologic tests varies widely but is typically 60-80% depending on the population tested. Specificity is estimated to be -98%. Because of the intermittent nature of colorectal bleeding, the sensitivity of FOBT and FIT is directly proportional to the number of samples taken and the frequency of testing.

Recent developments in testing look specifically for mutations in DNA characteristic of colorectal neoplasia that are detectable in exfoliated epithelial cells in the stool (Pignone, et al., 2002; Ahlquist, et al., 2002). While neoplastic bleeding is intermittent, epithelial shedding is continual, potentially making stool-based DNA testing (i.e., also known as fecal DNA [f-DNA] and stool DNA [sDNA]) testing more sensitive than other methods. Early studies of molecular fecal screening primarily focused on single mutations. Gene mutations in P53, K-ras, and BAT 26, for instance, have been linked to colorectal cancer and remain detectable in fecal samples. Colorectal neoplasms are varied in nature and no single mutation has been identified as being expressed universally. For this reason, multiple target assay panels (MTAP) are preferably used. PreGen-Plus™ (EXACT Sciences Corporation, Maynard, MA; Laboratory Corporation of America, Burlington, NC) is a single test that identifies the presence of 23 different microsatellite (MSI) mutations known to be associated with CRC, including mutations in BAT-26. Additionally, 21 other point mutations in other genes associated with CRC are included in this test: APC, K-ras, and p53. This test is further designed to detect long DNA fragments, which have been specifically associated with cells

called non-apoptotic colonocytes, which are common in CRC. While this test is more sensitive than fecal occult blood testing, it is not as sensitive as colonoscopy and will miss about half of cancers in an average risk group of people without symptoms.

Increased DNA methylation is an epigenetic alteration that is common in human cancers and is often associated with transcriptional silencing. Aberrantly methylated DNA has also been proposed as a potential tumor marker for CRC detection. Genes such as vimentin, which are transcriptionally silent in normal epithelium, have been considered as targets for cancer-associated aberrant methylation and for use as cancer markers (JNCI Journal of the National Cancer Institute 2005 97(15):1124-1132). A combined assay utilizing hypermethylated vimentin gene (hV) and a two site DNA integrity assay (DY), demonstrated a sensitivity of 88% for CRC with a specificity of 82% (Am J Gastroenterol. 2008 Nov; 103(11):2862-70). Further, ColoSure® is a single marker laboratory developed, stool based DNA test. This method examines DNA in exfoliated colon cells for cancer-associated aberrant methylation of the vimentin gene and reaches a sensitivity range of 72-77% and a specificity range of 83-94% in average risk individuals.

Protein tests provide an alternative method for detecting CRC. Tests assessing the presence of tumor-derived enzymes such as M2 pyruvate kinase (M2-PK), and/or proteins such as calprotectin, carcinoembryonic antigen (CEA), tissue inhibitor of metalloproteinase-1 (TIMP-1) and S100 calcium binding protein A12 (S100A12) have been described. A diagnosis of colorectal cancer using a combination of fecal occult blood and novel fecal protein markers S100A12 and TIMP-1 has been described in Clin Gastroenterol Hepatol. 2008 Oct;6(10):1122-8. Dimeric isoenzyme of pyruvate kinase, M2-PK, expressed by tumor cells, has as well been proposed as a screening tool for CRC. The performance of fecal M2-PK has been evaluated with IFOBT and colonoscopy in Am J Gastroenterol. 2008 Jun;103(6):1496-504. Compared to immunochemical FOBTs, TuM2-PK does not have supplemental value for screening for CRC because of a lower sensitivity and specificity (Eur J Gastroenterol Hepatol. 2007 Oct;19(10):878-82)

Although combined assays for detecting CRC have been described, their approach targets either multiple protein markers or either multiple DNA alterations. To date,

immunochemical tests and DNA tests for CRC detection have been evaluated and compared on a separate basis only.

EP0308227 describes a chemical fecal occult blood test employing a guaiac matrix.

5 EP0032782 describes a method for the detection of haemoglobin or decomposition products of haemoglobin in feces by means of an immunological reaction by using an antibody specific for human haemoglobin.

US7288413 describes methods that combine a chemical fecal occult blood test and an immunochemical fecal occult blood test.

10 WO 04/092709 concerns a fecal blood test involving the dispersement of a dye in toilet water.

EP0817968 describes several suitable stool collecting and testing methods and devices.

15 WO 05/017207 discloses that the vimentin gene can be a common target for methylation and epigenetic gene silencing in colon neoplasia, and may function as a candidate tumor suppressor gene.

WO 2008/084219 relates to detection of colorectal cancer based upon determining methylation of a number of different genes, including panels of genes.

WO 2006/113671 and WO 2008/010975 describe methylation markers relevant to colorectal cancer.

20 **SUMMARY OF THE INVENTION**

The invention provides a method of detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising:

(a) detecting the presence of blood in the faecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer,

(b) detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer

5 and based upon a positive result obtained in either (a) or (b) or in both (a) and (b) detecting a predisposition to, or the incidence of, colorectal cancer.

Also described herein is a method of sample processing, prior to carrying out a method of the invention, comprising removing a portion of a collected faecal sample and adding the removed portion of the sample to a buffer which prevents denaturation or degradation of blood proteins found in the sample.

10 The invention also provides a method of detecting a predisposition to, or the incidence of, colorectal cancer in a sample comprising detecting an epigenetic modification in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 , wherein detection of the epigenetic modification in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

15 The invention also provides a method of detecting a predisposition to, or the incidence of, cancer (and in particular colorectal cancer) in a sample comprising detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1, wherein detection of the epigenetic modification in the at least one gene is indicative of a predisposition to, or the incidence of, cancer (and in particular colorectal cancer).

20 The invention also relates to a method of detecting a predisposition to, or the incidence of, colorectal cancer (in particular in a faecal sample) comprising detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2, wherein detection of the epigenetic modification in the at least one gene is indicative of a predisposition to, or the incidence of, colorectal cancer.

25 In related aspects, the invention provides

a method for predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in:

(a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1,

5 (b) at least one gene selected from LAMA1 and CDO1; or

(c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein detection of the epigenetic modification in at least one of the genes in the panel or in the at least one gene is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

10 a method for predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in

(a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 ,

(b) at least one gene selected from LAMA1 and CDO1; or

15 (c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein detection of the epigenetic modification in at least one of the genes in the panel or in the at least one gene is indicative that the likelihood of resistance to treatment is lower than if the epigenetic modification is not detected.

20 a method of selecting a suitable treatment regimen for colorectal cancer comprising detecting an epigenetic modification in

(a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 ,

(b) at least one gene selected from LAMA1 and CDO1; or

(c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein detection of the epigenetic modification in at least one of the genes in the panel or in the at

least one gene results in selection of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor for treatment and wherein if the epigenetic modification is not detected, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment.

5 a method for monitoring treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in

(a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE 1 ,

(b) at least one gene selected from LAMA1 and CDO1 ; or

10 (c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein detection of a reduction in the epigenetic modification in at least one of the genes in the panel or in the at least one gene as treatment progresses is indicative of successful treatment.

15 Thus, the epigenetic modification may be measured at the start of the treatment and then once or more following treatment, or as treatment progresses, in order to determine if the treatment is achieving the desired effect. A return to lower levels of methylation of the genes is considered indicative of effective treatment.

The invention also relates to a kit for detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising:

20 (a) means for detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer, and

(b) means for detecting the presence of blood in the faecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer.

25

Also provided is a kit for any of:

- (a) detecting a predisposition to, or the incidence of, colorectal cancer in a sample
- (b) monitoring treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor
- 5 (c) predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor
- (d) predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor;
or
- 10 (e) selecting a suitable treatment regimen for colorectal cancer comprising means for detecting an epigenetic modification in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1.

Similarly, the invention also provides a kit for any of:

- (a) detecting a predisposition to, or the incidence of, colorectal cancer in a sample
- 15 (b) predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor
- (c) predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor;
or
- 20 (d) selecting a suitable treatment regimen for colorectal cancer comprising means for detecting an epigenetic modification in at least one gene selected from LAM A1 and CD01.

The invention also provides a kit for any of:

- (a) detecting a predisposition to, or the incidence of, colorectal cancer in a sample

(b) predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor

(c) predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor;
5 or (d) selecting a suitable treatment regimen for colorectal cancer comprising means for detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2 and means for processing a faecal sample.

The invention also provides a method of detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising detecting an epigenetic
10 modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer, characterised in that the faecal sample has previously been stored for at least approximately 6 months, 1, 2, 3, 4, 5, 6 or more years and/or is less than approximately 4, 3, 2, or 1g in weight.

15 DETAILED DESCRIPTION OF THE INVENTION

The invention, as set out in the claims, is based upon successful attempts to improve the detection of colorectal cancer. In particular, the invention aims to improve the positive and negative predictive value and also the sensitivity and specificity of detection of
20 colorectal cancer through non-invasive means. The methods of the invention may permit effective detection of colorectal cancer without the requirement for relatively expensive, highly invasive and painful procedures such as digital rectal examination, colonoscopy and sigmoidoscopy to be performed. The invention is based upon a combination of tests for detecting proteins and epigenetic modification markers respectively in the same faecal
25 sample, shown for the first time herein to provide a particularly useful overall test.

Thus, according to a first aspect, the invention provides a method of detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising, consisting essentially of or consisting of:

(a) detecting the presence of blood in the faecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer,

(b) detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer

and based upon a positive result obtained in either (a) or (b) or in both (a) and (b) detecting a predisposition to, or the incidence of, colorectal cancer.

As shown herein, the combination of methylation marker assay and fecal occult blood test (FOBT) gives very specific and sensitive results.

The combined methods of the invention improve the negative predictive value of the existing single tests. By improving sensitivity, the number of false negative results is decreased and this improves negative predictive value.

Step (a) of the methods involves detecting the presence of blood in the faecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer. Blood in the faeces is an indicator of intestinal bleeding, which is frequently associated with colorectal cancer. Thus, detection of blood in the faecal sample is considered a "positive" result in step (a). Any suitable method for detecting the presence of blood in the sample may be employed. Often, the methods of detecting blood will rely upon detecting a representative blood protein in the faecal sample. In certain embodiments, detecting the presence of blood in the faecal sample comprises, consists essentially of or consists of detection of haemoglobin in the faecal sample. Detection may be through any suitable means, and includes all variants of fecal occult blood tests. The test may be chromogenic or immunological in certain embodiments. The test may rely upon peroxidase activity of hemoglobin. Chromogenic tests are well known and commercially available and may rely upon chemical reagents such as gum guaiac. In specific embodiments, haemoglobin in the faecal sample is detected through immunochemical means. This may involve anti-hemoglobin antibodies in certain embodiments. The term "antibody" or "antibodies" herein refers to an antibody or antibodies, or a derivative thereof that retains

specific binding activity. By specific binding activity is meant the ability to specifically bind to hemoglobin. Thus, such a reagent does not bind, or does not bind to a significant degree, to unrelated proteins found in the faecal sample. Any antibody or derivative may be employed. Thus, the antibody may be a monoclonal or polyclonal antibody. The derivative of the antibody that retains specific binding activity may comprise, consist essentially of or consist of a humanized version of a non-human antibody, a heavy chain antibody, a single domain antibody, a nanobody, a Fab fragment or scFv etc. in certain embodiments. Numerous techniques are available for producing antibodies and their derivatized forms, as would be well known to one skilled in the art.

As mentioned above, the combination of techniques maximises sensitivity of detection, without significantly compromising specificity. Thus, the threshold detection concentrations for detection of blood/hemoglobin in step (a) may be those typically employed in fecal occult blood tests. Adding in the step (b) test improves overall sensitivity by picking up additional positive samples. For example, in some embodiments, the result in step (a) is considered positive if the concentration of hemoglobin detected is more than between (about) 50 to (about) 150 ng/ml. In more specific embodiments, the result in step (a) is considered positive if the concentration of hemoglobin detected is more than (about) 100 ng/ml.

However, in other embodiments, the methods of the invention may be employed to improve the sensitivity of the step (a) method, whilst preventing a resultant loss in specificity. By lowering the threshold concentration of blood to be detected in the faecal sample to give a positive result in step (a), the sensitivity of the step (a) method is increased. In order to retain specificity, the step (b) method is employed on those samples in which low levels, that is to say lower than the typically used threshold, of blood were detected in step (a). A positive result from the step (b) method is required to confirm the positive result in step (a) for the "low level" samples. For those samples having blood (especially hemaglobin) concentrations above the typically employed threshold in step (a), it is not necessary to perform the method of step (b), since for these samples the step (a) method is sufficiently specific for this not to be necessary. This has the advantage that the step (b) test is not required for all samples, thus reducing costs and increasing throughput. Thus, in certain

embodiments, the result in step (a) is considered positive if the concentration of hemoglobin detected is lower than is typically employed as the threshold concentration of hemoglobin in hemoglobin detection tests, but for those samples in which a "lower than typical threshold" concentration of hemoglobin is detected, step (b) is performed on these samples. The
5 detection of the epigenetic modification in step (b) is then used to confirm the positive result in step (a). The step (b) test is not employed for those samples in which the concentration of hemoglobin detected is higher than the threshold typically employed in hemoglobin detection tests.

In specific embodiments, the result in step (a) is considered positive if the
10 concentration of hemoglobin detected is more than or at least (about) 5 to (about) 50 ng/ml, more specifically more than or at least (about) 5 to (about) 20 ng/ml and more particularly more than or at least (about) 10 ng/ml. By lowering the threshold, the sensitivity of the test is increased. In such embodiments, step (b) is performed only in the event that the
15 concentration of hemoglobin detected is between (about) 5 ng/ml and (about) 250 ng/ml, more specifically between (about) 10 ng/ml and (about) 200 ng/ml. The detection of the epigenetic modification in step (b) is then used to confirm the positive result in step (a). Thus, a positive result in step (b) confirms the result in step (a) as positive. If no epigenetic modification of the DNA is detected, the result of step (a) is considered negative. For
20 samples in which the concentration of hemoglobin detected is more than or at least (about) 200 ng/ml (or (about) 250 ng/ml), it is not necessary to perform step (b), since the result in step (a) will be of high specificity (i.e. is unlikely to be a false positive).

Step (b) involves detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer. Thus, detection of the epigenetic
25 modification is considered a "positive" result in step (b).

In some embodiments, the epigenetic modification is detected in at least one gene selected from PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1 , LAMA1 , MMP2, OSMR, SFRP2 and CDO1, with detection of the epigenetic modification

in at least one of the genes providing an indication of a predisposition to, or incidence of, colorectal cancer.

In certain embodiments, the epigenetic modification is detected in at least one gene selected from PHACTR3, NDRG4 and FOXE1 , with detection of the epigenetic
5 modification in at least one of the genes providing an indication of a predisposition to, or incidence of, colorectal cancer.

PHACTR3 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 20 (location 20q13.32-q13.33) and the gene sequence is listed under the accession numbers AJ311122 and NM_080672. The gene
10 encodes the phosphatase and actin regulator 3.

NDRG4 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 16 (location q21-q22.3) and the gene sequence is listed under the accession number AB044947. The gene encodes NDRG family member 4.

FOXE1 is the gene symbol approved by the HUGO Gene Nomenclature Committee.
15 The gene is located on chromosome 9 (location 9q22) and the gene sequence is listed under the accession number U89995. The gene encodes the forkhead box E1 (thyroid transcription factor 2).

GATA4 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 8 (location 8p23.1-p22) and the gene sequence is listed
20 under the accession numbers AK097060 and NMJ302052. The gene encodes the GATA binding protein 4.

GPNMB is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 7 (location 7p) and the gene sequence is listed under the accession numbers X76534 and NMJ301005340. The gene encodes the glycoprotein
25 (transmembrane) nmb.

TFPI2 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 7 (location 7q) and the gene sequence is listed under the accession numbers L27624 and NM_006528. The gene encodes tissue factor pathway inhibitor 2.

5 SOX17 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 8 (location 8q11.23) and the gene sequence is listed under the accession number AB073988. The gene encodes SRY (sex determining region Y)-box 17.

10 SYNE1 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 6 (location 6q24.2-q25.3) and the gene sequence is listed under the accession number AB018339. The gene encodes spectrin repeat containing, nuclear envelope 1.

15 LAMA1 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 18 (location 18p11.3) and the gene sequence is listed under the accession numbers X58531 and NM_005559. The gene encodes laminin, alpha 1.

MMP2 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 16 (location 16q13-q21) and the gene sequence is listed under the accession number NM_001127891. The gene encodes matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase).

20 OSMR is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 5 (location 5p13.2) and the gene sequence is listed under the accession number U60805 and NM_003999. The gene encodes the oncostatin M receptor.

25 SFRP2 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 4 (location 4q31.3) and the gene sequence is listed under the accession number AF017986. The gene encodes the secreted frizzled-related protein 2.

CDO1 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 5 (location 5q23.2) and the gene sequence is listed under the accession number NM_001801. The gene encodes the cysteine dioxygenase, type I.

5 By "gene" is meant the specific known gene in question. It may also relate to any gene which is taken from the family to which the named "gene" belongs, in certain circumstances, and includes according to all aspects of the invention not only the particular sequences found in the publicly available database entries, but also encompasses transcript and nucleotide variants of these sequences, with the proviso that methylation or another epigenetic modification of the gene is linked to the incidence of colorectal cancer. Variant
10 sequences may have at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% nucleotide sequence identity with the nucleotide sequences in the database entries. Computer programs for determining percentage nucleotide sequence identity are available in the art, including the Basic Local Alignment Search Tool (BLAST) available from the National Center for
15 Biotechnology Information.

The methods of the invention are generally in vitro methods carried out on an isolated test sample. The methods are used to diagnose colorectal cancer. In certain embodiments the methods may also include the step of obtaining the sample. The test sample is generally from a human subject. The subject may be suspected of being tumorigenic. More specifically the
20 subject may be suspected of suffering from a colorectal cancer.

In specific embodiments of the methods, the epigenetic modification is detected in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 , with detection of the epigenetic modification in at least one of the genes providing an indication of a predisposition to, or incidence of, colorectal cancer. Specific panels which may be employed
25 comprise, consist essentially of or consist of PHACTR3, NDRG4 and FOXE1, NDRG4 and FOXE1 , PHACTR3 and NDRG4, or PHACTR3 and FOXE1.

In other embodiments, the epigenetic modification is detected in at least one gene selected from LAMA1 and CDO1. In further embodiments, the epigenetic modification is

detected in at least one gene selected from GPNMB and MMP2. The methods of the invention may have diagnostic and prognostic value, and this is included within the definition of the term "detecting a predisposition to, or the incidence of, colorectal cancer". The prognostic value of the methods of the invention may be used as a marker of potential susceptibility to colorectal cancer or as a marker for progression from adenoma to cancer for example. Thus patients at risk may be identified before the disease has a chance to manifest itself in terms of symptoms identifiable in the patient. Initial detection as well as follow-up detection, for example following treatment, is also included within the definition. Follow up detection may be performed after any treatment. Examples include following polypectomy (removal of polyps) and following surgery to remove a colorectal carcinoma, severe neoplasia or advanced adenoma.

The methods of the invention may be carried out on purified or unpurified DNA-containing samples. However, in specific embodiments, DNA is isolated/extracted/purified from the sample. Any suitable DNA isolation technique may be utilised. Examples of purification techniques may be found in standard texts such as Molecular Cloning - A Laboratory Manual (Third Edition), Sambrook and Russell (see in particular Appendix 8 and Chapter 5 therein). In some embodiments, purification involves alcohol precipitation of DNA. Preferred alcohols include ethanol and isopropanol. Suitable purification techniques also include salt-based precipitation methods. Thus, in specific embodiments the DNA purification technique comprises use of a high concentration of salt to precipitate contaminants. The salt may comprise, consist essentially of or consist of potassium acetate and/or ammonium acetate for example. The method may further include steps of removal of contaminants which have been precipitated, followed by recovery of DNA through alcohol precipitation.

In alternative embodiments, the DNA purification technique is based upon use of organic solvents to extract contaminants from cell lysates. Thus, in certain embodiments, the method comprises use of phenol, chloroform and isoamyl alcohol to extract the DNA. Suitable conditions are employed to ensure that the contaminants are separated into the organic phase and that DNA remains in the aqueous phase.

In specific embodiments of these purification techniques, extracted DNA is recovered through alcohol precipitation, such as ethanol or isopropanol precipitation.

Amplification of DNA (using PCR) from natural sources is often inhibited by co-purified contaminants and various methods adopted for DNA extraction from environmental samples are available and provide an alternative for isolating DNA from faecal samples, according to specific embodiments of the invention. For instance, the QIAamp DNA Stool Mini Kit from QIAGEN adsorbs DNA-damaging substances and PCR inhibitors present in the sample by InhibitEX. Other examples for application to faecal samples include the Wizard Genomic DNA Purification Kit (Promega), the NucliSENS® easyMAG™ (Biomerieux) and nucleic acid purification kits manufactured by Macherey Nagel.

The methods of the invention may also, as appropriate, incorporate quantification of isolated/extracted/purified DNA in the sample. Quantification of the DNA in the sample may be achieved using any suitable means. Quantitation of nucleic acids may, for example, be based upon use of a spectrophotometer, a fluorometer or a UV transilluminator. Examples of suitable techniques are described in standard texts such as Molecular Cloning - A Laboratory Manual (Third Edition), Sambrook and Russell (see in particular Appendix 8 therein). In some embodiments, kits such as the Picogreen® dsDNA quantitation kit available from Molecular Probes, Invitrogen may be employed to quantify the DNA.

The inventor has determined that faecal samples for use in the invention do not necessarily have to be freshly collected. This applies in particular for performing step (b) and thus also the further methods of the invention discussed herein where epigenetic modifications in specific genes are determined in faecal samples. Thus, samples collected 1, 2, 3, 4, 5, 6 or more years ago may be employed. The historical samples may have been frozen for storage. Freezing is performed at a suitable temperature, such as 20°C for example. Lyophilisation may be performed in certain embodiments to permit storage of the faecal samples. The sample may be frozen without the addition of a stabilizing buffer in certain embodiments as it has been shown that this is not necessary, when the sample is frozen, in order to retain DNA integrity in the sample. In other embodiments, a suitable

stabilizing buffer as known to those in the art, may be added to the sample before and/or after storage.

The inventor has also discovered that a minimal stool sample, including a minimal sample derived from a historical sample as discussed herein, may successfully be employed in the methods of the invention. This is particularly applicable to performing step (b) and thus is also applicable to the further aspects of the invention discussed herein where epigenetic modifications in specific genes are determined, in faecal samples. Thus, only a portion of a collected faecal sample need be employed in the methods of the invention in order to achieve reliable results. Reliable results may be achieved by isolating, extracting or purifying DNA from less than 5g of faecal sample, in particular from approximately 4, 3, 2, or 1g of faecal sample. The faecal sample may be present in an appropriate volume of homogenizing buffer to facilitate DNA isolation, extraction and purification.

As discussed above, suitable commercially available kits, such as the QIAamp DNA Stool Mini Kit, may be employed in order to extract the DNA and remove potential inhibitors found in the faecal sample. This may involve processing smaller volumes of the overall faecal sample and pooling the resultant DNA in certain embodiments. For example, volumes of approximately 1ml or less, such as approximately 750, 500, 250 or 100 µl may be used for DNA extraction. The resultant DNA from 2, 3, 4, 5 or more extractions may be pooled to give an overall DNA sample to be assessed according to the methods of the invention. DNA in the sample, pre- and/or post-pooling can be quantified as required, as discussed herein.

Thus, the invention provides a method of detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising, consisting essentially of or consisting of detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer, characterised in that the faecal sample has previously been stored for at least approximately 6 months, 1, 2, 3, 4, 5, 6 or more years and/or is less than approximately 4, 3, 2, or 1g in weight (prior to addition of homogenization buffer).

The detailed discussion of determination of epigenetic modifications provided herein applies mutatis mutandis to this aspect of the invention.

"Colorectal cancer", also called colon cancer or bowel cancer, is defined to include cancerous growths, carcinomas, severe neoplasias and advanced adenomas in the colon, rectum and appendix.

"Epigenetic modification" is defined herein to include 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. Epigenetic modifications may, in certain circumstances be stable heritable traits. A number of related mechanisms that cause alteration in gene expression are recognised and include DNA methylation, histone changes (for example changes in histone acetylation) which may lead to chromatin remodelling and RNA interference. In many cases, hypermethylation of DNA incorrectly switches off critical genes allowing cancers to develop and progress. In specific embodiments, the epigenetic modification is methylation. In particular, aberrant methylation, which may be referred to as hypermethylation, of the gene or genes is detected. Typically, the methylation status is determined in suitable CpG islands which are often found in the promoter region of the gene(s). The term "methylation", "methylation state" or "methylation status" refers to the presence or absence of 5-methylcytosine at one or a plurality of CpG dinucleotides within a DNA sequence. CpG dinucleotides are typically concentrated in the promoter regions and exons of human genes. CpG dinucleotides susceptible to methylation may be found in the promoter region, exons and introns of human genes. Promoter, exon and intron regions can all thus be assessed for methylation. A "promoter" is a region extending typically between approximately 1 Kb, 500 bp or 150 to 300 bp upstream from the transcription start site. In some embodiments, the CpG island surrounding or positioned around the transcription start site is analysed to determine its methylation status. Alternatively, the methylation status of the exon and/or intron regions of may be determined as appropriate. The identification of CpG islands, to assess for determination of methylation status, is a matter of routine for one skilled in the art and various techniques, including in silico techniques, are available.

Since hypermethylation of a gene may result in diminished gene expression it may be possible, in certain embodiments, to carry out the methods of the invention by determining gene expression of the DNA in the faecal sample, rather than investigating the methylation status of the DNA directly. Many suitable techniques are available for determining gene expression at either the RNA or protein level. In certain embodiments, expression at the RNA level is determined by reverse transcriptase polymerase chain reaction (RT-PCR). In alternative embodiments, expression is determined at the protein level. Again, any suitable technique may be employed such as western blotting, ELISA etc.

Measurement of expression of a gene on its own may not necessarily conclusively indicate that the silencing is epigenetic, as the mechanism of silencing could be genetic, for example, by somatic mutation. Accordingly, in certain embodiments, the methods of the invention incorporate an appropriate re-expression assay which is designed to reverse epigenetic silencing. Appropriate treatment of the sample using a demethylating agent, such as a DNA-methyltransferase (DMT) inhibitor may reverse epigenetic silencing of the relevant gene. Suitable reagents include, but are not limited to, DAC (5'-deazacytidine), TSA or any other treatment affecting epigenetic mechanisms present in cell lines. Suitable reagents are discussed herein with respect to the pharmacogenetic, treatment monitoring and treatment aspects of invention, which discussion applies mutatis mutandis. Typically, expression is reactivated or reversed upon treatment with such reagents, indicating that the silencing is epigenetic.

Determination of the methylation status may be achieved through any suitable means. Suitable examples include bisulphite genomic sequencing and/or by methylation specific PCR. Various techniques for assessing methylation status are known in the art and can be used in conjunction with the present invention: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR (McMS-PCR), MLPA with or without bisulphite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulphite conversion-specific methylation-specific PCR (BS-MSP) (Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence differences in PCR products of sodium bisulphite - treated DNA), methylation-sensitive single-nucleotide primer

extension conformation(MS-SNuPE), methylation- sensitive single-strand conformation analysis (MS-SSCA), Melting curve combined bisulphite restriction analysis (McCOBRA)(Akey et al., 2002), PyroMethA, HeavyMethyl (Cottrell et al. 2004), MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles (QAMA), enzymatic regional methylation assay (ERMA)1 QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques for DNA methylation analysis is provided in Nucleic acids research, 1998, Vol. 26, No. 10, 2255-2264, Nature Reviews, 2003, Vol.3, 253-266; Oral Oncology, 2006, Vol. 42, 5-13, which references are incorporated herein in their entirety.

Techniques for assessing methylation status are based on distinct approaches. Some include use of endonucleases. Such endonucleases may either preferentially cleave methylated recognition sites relative to non-methylated recognition sites or preferentially cleave non-methylated relative to methylated recognition sites. Some examples of the former are Ace III, Ban I, BstN I, Msp I, and Xma I. Examples of the latter are Ace II, Ava I, BssH II, BstU I, Hpa II, and Not I. Differences in cleavage pattern are indicative for the presence or absence of a methylated CpG dinucleotide. Cleavage patterns can be detected directly, or after a further reaction which creates products which are easily distinguishable. Means which detect altered size and/or charge can be used to detect modified products, including but not limited to electrophoresis, chromatography, and mass spectrometry.

Alternatively, the identification of methylated CpG dinucleotides may utilize the ability of the methyl binding domain (MBD) of the MeCP2 protein to selectively bind to methylated DNA sequences (Cross et al, 1994; Shiraishi et al, 1999). The MBD may also be obtained from MBP, MBP2, MBP4, poly-MBD (Jorgensen et al., 2006) or from reagents such as antibodies binding to methylated nucleic acid. The MBD may be immobilized to a solid matrix and used for preparative column chromatography to isolate highly methylated DNA sequences. Variant forms such as expressed His-tagged methyl-CpG binding domain may be used to selectively bind to methylated DNA sequences. Eventually, restriction endonuclease digested genomic DNA is contacted with expressed His-tagged methyl-CpG binding domain. Other methods are well known in the art and include amongst others methylated-CpG island recovery assay (MIRA). Another method, MB- PCR, uses a

recombinant, bivalent methyl-CpG-binding polypeptide immobilized on the walls of a PCR vessel to capture methylated DNA and the subsequent detection of bound methylated DNA by PCR.

Further approaches for detecting methylated CpG dinucleotide motifs use chemical reagents that selectively modify either the methylated or non-methylated form of CpG dinucleotide motifs. Suitable chemical reagents include hydrazine and bisulphite ions. The methods of the invention preferably use bisulphite ions. The bisulphite conversion relies on treatment of DNA samples with sodium bisulphite which converts unmethylated cytosine to uracil, while methylated cytosines are maintained (Furuichi et al., 1970). This conversion finally results in a change in the sequence of the original DNA. It is general knowledge that the resulting uracil has the base pairing behaviour of thymidine which differs from cytosine base pairing behaviour. This makes the discrimination between methylated and non-methylated cytosines possible. Useful conventional techniques of molecular biology and nucleic acid chemistry for assessing sequence differences are well known in the art and explained in the literature. See, for example, Sambrook, J., et al., *Molecular cloning: A laboratory Manual*, (2001) 3rd edition, Cold Spring Harbor, NY; Gait, M.J.(ed.), *Oligonucleotide Synthesis, A Practical Approach*, IRL Press (1984); Hames B.D., and Higgins, S.J. (eds.), *Nucleic Acid Hybridization, A Practical Approach*, IRL Press (1985); and the series, *Methods in Enzymology*, Academic Press, Inc.

Some techniques use primers for assessing the methylation status at CpG dinucleotides. Two approaches to primer design are possible. Firstly, primers may be designed that themselves do not cover any potential sites of DNA methylation. Sequence variations at sites of differential methylation are located between the two primers and visualisation of the sequence variation requires further assay steps. Such primers are used in bisulphite genomic sequencing, COBRA, Ms-SnuPE and several other techniques. Secondly, primers may be designed that hybridize specifically with either the methylated or unmethylated version of the initial treated sequence. After hybridization, an amplification reaction can be performed and amplification products assayed using any detection system known in the art. The presence of an amplification product indicates that a sample hybridized to the primer. The specificity of the primer indicates whether the DNA had been modified or

not, which in turn indicates whether the DNA had been methylated or not. If there is a sufficient region of complementarity, e.g., 12, 15, 18, or 20 nucleotides, to the target, then the primer may also contain additional nucleotide residues that do not interfere with hybridization but may be useful for other manipulations. Examples of such other residues
5 may be sites for restriction endonuclease cleavage, for ligand binding or for factor binding or linkers or repeats. The oligonucleotide primers may or may not be such that they are specific for modified methylated residues.

A further way to distinguish between modified and unmodified nucleic acid is to use oligonucleotide probes. Such probes may hybridize directly to modified nucleic acid or to
10 further products of modified nucleic acid, such as products obtained by amplification. Probe-based assays exploit the oligonucleotide hybridisation to specific sequences and subsequent detection of the hybrid. There may also be further purification steps before the amplification product is detected e.g. a precipitation step. Oligonucleotide probes may be labelled using
15 any detection system known in the art. These include but are not limited to fluorescent moieties, radioisotope labelled moieties, bioluminescent moieties, luminescent moieties, chemiluminescent moieties, enzymes, substrates, receptors, or ligands.

In the MSP approach, DNA may be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulphite treatment (Herman et al., 1996; and WO 97/46705). For
20 example, bisulphite ions modify non-methylated cytosine bases, changing them to uracil bases. Uracil bases hybridize to adenine bases under hybridization conditions. Thus an oligonucleotide primer which comprises adenine bases in place of guanine bases would hybridize to the bisulphite-modified DNA, whereas an oligonucleotide primer containing the guanine bases would hybridize to the non-modified (methylated) cytosine residues in the
25 DNA. Amplification using a DNA polymerase and a second primer yield amplification products which can be readily observed, which in turn indicates whether the DNA had been methylated or not. Whereas PCR is a preferred amplification method, variants on this basic technique such as nested PCR and multiplex PCR are also included within the scope of the invention.

As mentioned earlier, a preferred embodiment for assessing the methylation status of the relevant gene requires amplification to yield amplification products. The presence of amplification products may be assessed directly using methods well known in the art. They simply may be visualized on a suitable gel, such as an agarose or polyacrylamide gel.

5 Detection may involve the binding of specific dyes, such as ethidium bromide, which intercalate into double-stranded DNA and visualisation of the DNA bands under a UV illuminator for example. Another means for detecting amplification products comprises hybridization with oligonucleotide probes. Alternatively, fluorescence or energy transfer can be measured to determine the presence of the methylated DNA.

10 A specific example of the MSP technique is designated real-time quantitative MSP (QMSP), and permits reliable quantification of methylated DNA in real time or at end point. Real-time methods are generally based on the continuous optical monitoring of an amplification procedure and utilise fluorescently labelled reagents whose incorporation in a product can be quantified and whose quantification is indicative of copy number of that
15 sequence in the template. One such reagent is a fluorescent dye, called SYBR Green I that preferentially binds double-stranded DNA and whose fluorescence is greatly enhanced by binding of double-stranded DNA. Alternatively, labeled primers and/or labeled probes can be used for quantification. They represent a specific application of the well known and commercially available real-time amplification techniques such as TAQMAN®,
20 MOLECULAR BEACONS®, AMPLIFLUOR® and SCORPION® DzyNA®, Plexor™ etc. In the real-time PCR systems, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template.

25 In specific embodiments, methylation specific PCR/amplification is utilised. This may be carried out in real time or at end point. The real time or end point PCR/amplification may involve use of hairpin primers (Amplifluor), hairpin probes (Molecular Beacons), hydrolytic probes (Taqman), FRET probe pairs (Lightcycler), primers incorporating a hairpin probe (Scorpion), fluorescent dyes (SYBR Green etc.), primers incorporating the complementary sequence of a DNAzyme and a cleavable fluorescent DNAzyme substrate or
30 oligonucleotide blockers, for example.

Real-Time PCR detects the accumulation of amplicon during the reaction. Real-time methods do not need to be utilised, however. Many applications do not require quantification and Real-Time PCR is used only as a tool to obtain convenient results presentation and storage, and at the same time to avoid post-PCR handling. Thus, analyses can be performed only to confirm whether the target DNA is present in the sample or not. Such end-point verification is carried out after the amplification reaction has finished. This knowledge can be used in a medical diagnostic laboratory to detect a predisposition to, or the incidence of, cancer in a patient. End-point PCR fluorescence detection techniques can use the same approaches as widely used for Real Time PCR. For example, «Gene» detector allows the measurement of fluorescence directly in PCR tubes.

In real-time embodiments, quantitation may be on an absolute basis, or may be relative to a constitutively methylated DNA standard, or may be relative to an unmethylated DNA standard. Methylation status may be determined by using the ratio between the signal of the marker under investigation and the signal of a reference gene where methylation status is known (such as β -actin for example), or by using the ratio between the methylated marker and the sum of the methylated and the non-methylated marker. Alternatively, absolute copy number of the methylated marker gene can be determined.

Suitable controls may need to be incorporated in order to ensure the method chosen is working correctly and reliably. Suitable controls may include assessing the methylation status of a gene known to be methylated. This experiment acts as a positive control to ensure that false negative results are not obtained. The gene may be one which is known to be methylated in the sample under investigation or it may have been artificially methylated, for example by using a suitable methyltransferase enzyme, such as Sssl methyltransferase.

Additionally or alternatively, suitable negative controls may be employed with the methods of the invention. Here, suitable controls may include assessing the methylation status of a gene known to be unmethylated or a gene that has been artificially demethylated. This experiment acts as a negative control to ensure that false positive results are not obtained.

Whilst PCR is the preferred nucleic acid amplification technique, other amplification techniques may also be utilised to detect the methylation status of the concerned gene. Such amplification techniques are well known in the art, and include methods such as NASBA (Compton, 1991), 3SR (Fahy et al., 1991) and Transcription Mediated Amplification (TMA). Other suitable amplification methods include the ligase chain reaction (LCR) (Barringer et al, 1990), selective amplification of target polynucleotide sequences (US Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (US Patent No 4,437,975), arbitrarily primed polymerase chain reaction (WO 90/06995), invader technology, strand displacement technology, and nick displacement amplification (WO 2004/067726). This list is not intended to be exhaustive; any nucleic acid amplification technique may be used provided the appropriate nucleic acid product is specifically amplified. Thus, these amplification techniques may be tied in to MSP and/or bisulphite sequencing techniques for example.

In specific embodiments, the methods utilise primers selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth in table 1. Suitable primer pairs can be selected from the primers listed in the table based upon the gene or genes of interest. In specific embodiments, the methods utilise primers selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOXE1), and 7 and 8 (NDRG4) in order to detect methylation status in the DNA. These primers are particularly useful in MSP based methods of detection. Each of the primers covers one or more methylation sites and is specific for the methylated sequence following bisulphite treatment.

In certain embodiments, the methods utilise probes selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth in table 1.

Suitable probes can be selected from the probes listed in the table based upon the gene or genes of interest. In specific embodiments, the methods utilise probes selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOXE1), and 9 (NDRG4) in order to detect methylation status in the DNA. Each of the probes covers one or more methylation sites and is specific for the methylated sequence following bisulphite treatment. The probes may be utilised in

combination with the primers listed above in specific embodiments. The probes may be fluorescently labelled, in particular with a donor and an acceptor (or quencher) fluorophore to permit FRET to occur and allow real-time or end-point detection. An example of a donor moiety is FAM and an example of an acceptor or quencher moiety is DABCYL.

5 The methods of the invention are performed using a faecal sample. In certain embodiments, a single faecal sample is utilised as the source of the sample for (a) and (b). However, the sample may need to be split to permit (a) and (b) to be carried out. Thus the sample may need to be treated to differing degrees in order to permit the separate steps to be carried out. As a result, after obtaining the faecal sample, the sample may be apportioned
10 appropriately to allow the DNA and protein testing to be carried out separately.

 In the context of these methods of the invention, there is the requirement for appropriate sample processing, since two separate detection techniques may be performed on a single sample. To meet this requirement, the invention provides a method of sample processing, prior to carrying out a method of the invention (tests (a) and (b)), comprising
15 removing a portion of a collected faecal sample and adding the removed portion of the sample to a buffer which prevents denaturation or degradation of blood proteins found in the sample. The faecal sample may be collected at home by the subject. This may involve defecation into a suitable vessel. From this sample is removed a sufficient portion of the sample. This removed portion is then added to a suitable container, which may already
20 contain a buffer. Alternatively, the buffer may be added with the removed portion of the sample or (shortly) afterwards. The removed portion is thus prepared for performance of step (a), namely detecting the presence of blood in the faecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer, on this sample. The buffer prevents denaturation or degradation of blood proteins found in
25 the sample. Suitable buffers for protein preservation are known in the art and commercially available. The remaining portion of the sample may remain in the vessel in which it was originally collected, prior to carrying out step (b), namely detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal
30 cancer. Suitable buffers for maintaining DNA integrity may be added to the remaining

portion of the sample, in certain embodiments. The separated samples may be suitably labelled to ensure that they are paired up correctly during subsequent processing, including data analysis and comparison.

Following collection of the sample and suitable processing, the method may further
5 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. The forwarding or otherwise delivering may occur within any suitable time frame following sample collection. In certain embodiments, the forwarding or otherwise delivering may occur within 6, 12, 24, 48 or 72 hours of sample
10 collection. The method may then further comprise performing step (a) of the method on the removed portion of the collected faecal sample once it has been delivered. As described herein, step (b) may only be performed on a specific subset of samples in which blood is detected at low levels. Thus, the methods of sample processing may involve sorting those samples with detection of low levels of blood detected in step (a) (as defined herein) for
15 testing in step (b). The appropriate remaining portions of the collected samples in which step (b) is not required (i.e. where a confirmation of the result in step (a) is not required - either because the no blood is detected or the level of blood is above the typically employed threshold (such as 200 ng/ml)) may then be discarded as appropriate.

Similarly, following collection of the sample and suitable processing, the method may
20 further comprise forwarding or otherwise delivering the remaining portion of the collected faecal sample to a laboratory for performing step (b) of the method, as defined herein, on the remaining portion of the collected faecal sample. The forwarding or otherwise delivering may occur within any suitable time frame following sample collection. In certain
25 embodiments, the forwarding or otherwise delivering may occur within 6, 12, 24, 48 or 72 hours of sample collection. The method may then further comprise performing step (b) of the method on the remaining portion of the collected faecal sample once it has been delivered.

In certain embodiments, the forwarding or other delivery of the removed and remaining portions of the collected faecal sample may be carried out to the same location, optionally at the same time. This is the case where the same laboratory is carrying out both

steps (a) and (b) which may be the case where the laboratory has the capacity to perform both types of test.

As discussed herein, the faecal samples for use in the invention do not necessarily have to be freshly collected. The discussion above applies mutatis mutandis here. Similarly, a minimal stool sample, including a minimal sample derived from a historical sample as discussed herein, may successfully be employed in the methods of the invention. This is particularly applicable to performing step (b) (i.e. on the remaining portion of the faecal sample). Again, the discussion above applies mutatis mutandis here, including relating to pooling of samples during the DNA extraction process. The requirement for minimal or portioned samples for step (b) may be advantageous by permitting more of the sample to be utilised to perform step (a), potentially improving the sensitivity of step (a).

As shown herein, methylation status determination based upon a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 can provide sensitive and specific detection of colorectal cancer. Thus, according to a related aspect, the invention provides a method of detecting a predisposition to, or the incidence of, colorectal cancer in a sample comprising detecting an epigenetic modification in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1, wherein detection of the epigenetic modification in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

It is also shown herein that methylation of LAMA1 and CDO1 is linked to the incidence of colorectal cancer. These genes have not been previously linked to colorectal cancer. Thus, the invention also provides a method of detecting a predisposition to, or the incidence of, cancer (and in particular colorectal cancer) in a sample comprising detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1, wherein detection of the epigenetic modification in the at least one gene is indicative of a predisposition to, or the incidence of, cancer (and in particular colorectal cancer).

Evidence is also presented herein that methylation of GPNMB and MMP2 is linked to colorectal cancer, in particular based upon faecal samples. Thus, the invention also relates to

a method of detecting a predisposition to, or the incidence of, colorectal cancer (in particular in a faecal sample) comprising detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2, wherein detection of the epigenetic modification in the at least one gene is indicative of a predisposition to, or the incidence of, colorectal cancer.

5 As for the first aspect of the invention, the epigenetic modification is often methylation and thus these methods may involve determining the methylation status of the at least one gene or panel of genes. Thus, aberrant methylation, or "hypermethylation", of the gene(s) may be detected. Various methods for determining methylation status are discussed herein, which discussion applies mutatis mutandis to these aspects of the invention. In
10 specific embodiments, methylation specific PCR/amplification is utilised. This may be carried out in real time or at end point. The real time or end point PCR/amplification may involve use of hairpin primers (Amplifluor), hairpin probes (Molecular Beacons), hydrolytic probes (Taqman), FRET probe pairs (Lightcycler), primers incorporating a hairpin probe (Scorpion), fluorescent dyes (SYBR Green etc.), primers incorporating the complementary
15 sequence of a DNzyme and a cleavable fluorescent DNzyme substrate or oligonucleotide blockers, for example. The methods may apply suitable primers and probes selected from table 1. In specific embodiments, the methods utilise primers selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOX E1), and 7 and 8 (NDRG4). In further
20 embodiments, the methods utilise probes selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOX E1), and 9 (NDRG4). In other embodiments, the methods utilise primers selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 28 and 29 (LAMA1) and 34 and 35 (CDO1). In further related embodiments, the methods
25 utilise probes selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 30 (LAMA1) and 36 (CDO1). In other embodiments, the methods utilise primers selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 22 and 23 (GPNMB) and 31 and 32 (MMP2). In related embodiments, the methods utilise probes selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 24
30 (GPNMB) and 33 (MMP2).

These methods of the invention may be ex vivo or in vitro methods carried out on a test sample. The methods may be non-invasive. The methods may be used to identify any stage of colorectal cancer, including pre-malignancies such as adenomas right through to carcinomas (see the definition of colorectal cancer herein).

5 The "sample" in which the epigenetic modification is detected may comprise, consist essentially of or consist of a tissue sample, a faecal sample or a blood sample. In preferred embodiments, the sample is a faecal sample. Historical samples (such as those collected at least approximately 1 year ago) and small samples (such as those of approximately 1g) may be useful in the methods of the invention, as discussed herein (which discussion applies
10 mutatis mutandis). In specific embodiments, the tissue sample comprises, consists essentially of or consists of a colon and/or rectum and/or appendix sample. However, the sample may be from any representative tissue sample, body fluid, body fluid precipitate or lavage specimen, as required provided that detection of the epigenetic modification in the sample provides a reliable indicator of colorectal cancer. The sample may be obtained from a human subject.
15 Test samples for diagnostic, prognostic, or personalised medicinal uses can be obtained from surgical samples, such as biopsies or fine needle aspirates, from paraffin embedded tissues, from frozen tumor tissue samples, from fresh tumor tissue samples, from a fresh or frozen body fluid, for example. Non-limiting examples include whole blood, bone marrow, cerebral spinal fluid, peritoneal fluid, pleural fluid, lymph fluid, serum, plasma, urine, chyle, stool,
20 ejaculate, sputum, nipple aspirate, saliva, swabs specimen, wash or lavage fluid and/or brush specimens.

 These methods may also include the step of obtaining the test sample, in certain embodiments. The tissue sample or liquid sample comprising the nucleic acid may be lysed or need to be concentrated to create a mixture of biological compounds comprising nucleic
25 acids and other components. Alternatively, the nucleic acid may need to be cleared of proteins or other contaminants, e.g. by treatment with proteinase K. Procedures for lysing or concentrating biological samples are known by the person skilled in the art and can be chemical, enzymatic or physical in nature. A combination of these procedures may be applicable as well. For instance, lysis may be performed using ultrasound, high pressure,
30 shear forces, alkali, detergents or chaotropic saline solutions, or proteases or lipases. For the

lysis procedure to obtain nucleic acids, or concentrating nucleic acid from samples, reference may be made to Sambrook, J., et al., Molecular cloning: A Laboratory Manual, (2001) 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York Ausubel, F.M., et al., Current Protocols in Molecular Biology (1987), J. Wiley and Sons, New York.

5 The test sample is generally obtained from a (human) subject suspected of being tumorigenic. Alternatively the test sample is obtained from a subject undergoing routine examination and not necessarily being suspected of having a disease. Thus patients at risk can be identified before the disease has a chance to manifest itself in terms of symptoms identifiable in the patient.

10 Alternatively the sample is obtained from a subject undergoing treatment, or from patients being checked for recurrence of disease.

 Cancers characterised by hypermethylation of tumour suppressor genes may be treated by reducing methylation within the tumour cells. Various agents may be employed for this purpose, including DNA demethylating agents, DNA methyltransferase inhibitors and
15 HDAC inhibitors. Accordingly, the invention also provides a method for predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in: (a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 ,

20 (b) at least one gene selected from LAMA1 and CDO1; or

 (c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein detection of the epigenetic modification in at least one of the genes in the panel or in the at least one gene is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

25 Similarly, the invention provides a method for predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic

modification in (a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 ,

(b) at least one gene selected from LAMA1 and CDO1; or

(c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein
5 detection of the epigenetic modification in at least one of the genes in the panel or in the at least one gene is indicative that the likelihood of resistance to treatment is lower than if the epigenetic modification is not detected.

Furthermore, there is provided a method of selecting a suitable treatment regimen for colorectal cancer comprising detecting an epigenetic modification in (a) a panel of at least
10 two genes selected from PHACTR3, NDRG4 and FOXE1, (b) at least one gene selected from LAMA1 and CDO1 ; or

(c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) wherein
detection of the epigenetic modification in at least one of the genes in the panel or in the at least one gene results in selection of a DNA demethylating agent and/or a DNA
15 methyltransferase inhibitor and/or a HDAC inhibitor for treatment and wherein if the epigenetic modification is not detected, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment.

In a related aspect, the invention provides a method for monitoring treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor
20 and/or HDAC inhibitor comprising detecting an epigenetic modification in

(a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1,

(b) at least one gene selected from LAMA1 and CDO1 ; or (c) at least one gene
selected from GPNMB and MMP2 (in a faecal sample) wherein detection of a reduction in
the (levels of the) epigenetic modification in at least one of the genes in the panel or in the at
25 least one gene as treatment progresses is indicative of successful treatment. Thus, the epigenetic modification may be measured at the start of the treatment and then once or more

following treatment, or as treatment progresses, in order to determine if the treatment is achieving the desired effect. A return to lower levels of methylation of the genes is considered indicative of effective treatment. Thus, the method may involve testing samples taken at various time points in order to determine the effect of the treatment. A threshold may
5 be set (i.e. a level of reduction in the epigenetic modification) at which treatment is considered successful and may, therefore, be reduced or terminated.

For all of the relevant methods (pharmacogenetic methods, treatment regimen methods, monitoring methods and methods of treatment) of the invention, the DNA demethylating agent may be any agent capable of alleviating hypermethylation of the
10 relevant genes (and thus up regulating transcription of the genes). The DNA methyltransferase inhibitor may be any suitable inhibitor of DNA methyltransferase activity or expression which is suitable for treating cancer in the presence of methylation of the at least one gene. The DNA methyltransferase inhibitor may, be one which reduces expression of DNMT genes, such as suitable antisense molecules, or double stranded RNA molecules,
15 such as siRNA and miRNA molecules which mediate RNAi for example. The design of a suitable siRNA molecule is within the capability of the skilled person and suitable molecules can be made to order by commercial entities (see for example, www.ambion.com). In embodiments, the DNA methyltransferase gene is (human) DNMT1.

Alternatively, the agent may be a direct inhibitor of DNMTs. Examples include
20 modified nucleotides such as phosphorothioate modified oligonucleotides (fig 6 of Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31) and nucleosides and nucleotides such as cytidine analogues. Suitable examples of cytidine analogues include 5-azacytidine, 5-aza-2'-deoxycytidine, 5-fluouoro-2'-deoxycytidine, pseudoisocytidine, 5,6-
25 dihydro-5-azacytidine, 1- β -D- arabinofuranosyl-5-azacytosine (known as fazabarine) (see figure 4 of Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31). The DNA methyltransferase inhibitor may comprise Decitabine.

Additional DNMT inhibitors include S-Adenosyl-Methionine (SAM) related compounds like ethyl group donors such as L-ethionine and non-alkylating agents such as S-adenosyl-homocysteine (SAH), sinefungin, (S)-6-methyl-6-deaminosine fungin, 6-deaminosinefungin, N4-adenosyl-N4-methyl-2,4-diaminobutanoic acid, 5'-methylthio-5'-deoxyadenosine (MTA) and 5'-amino-5'-deoxyadenosine (Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31). Useful DNMT inhibitors in the present invention comprise, consists essentially of or consists of 5-azacytidine and/or zebulaine.

Further agents which may alter DNA methylation and which may, therefore, be useful in the present invention as DNA demethylating agents include organohalogenated compounds such as chloroform etc, procianamide, intercalating agents such as mitomycin C, 4-aminobiphenyl etc, inorganic salts of arsenic and selenium and antibiotics such as kanamycin, hygromycin and cefotaxim (Villar-Garea, A. And Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Current Drug Metabolism, 2003, 4, 11-31).

Many HDAC inhibitors are similarly known in the art. Examples include carboxylic acid based HDAC inhibitors such as valproate and/or butyrate and hydroxamic acid based HDAC inhibitors such as trichostatin A, suberoyl hydroxamic acid (SBHA), 6-(3-chlorophenylureido)caproic hydroxamic acid (3-CI-UCHA), m-carboxycinnamic acid bishydroxylamide (CBHA), suberoylanilide hydroxamic acid (SAHA), azelaic bishydroxamic acid (ABHA), pyroxamide, aromatic sulfonamides bearing a hydroxamic acid group and cyclic-hydroxamic-acid containing peptides.

For each of these additional aspects, the embodiments and optional features of the methods of the invention apply mutatis mutandis and are not repeated for reasons of conciseness. Thus, all methods of detecting an epigenetic modification in the DNA, in particular in the specified genes, including expression and re-expression based assays, may be employed appropriately.

In a further related aspect, the invention provides a method of treating colorectal cancer in a subject comprising administration of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor wherein the subject has been selected for treatment on the basis of a method of the invention. Thus, detecting an epigenetic modification in

(a) a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 ,

(b) at least one gene selected from LAMA1 and CDO1; or

(c) at least one gene selected from GPNMB and MMP2 (in a faecal sample) may be used in order to direct treatment of the subject (from which the sample was taken).

The invention also relates to corresponding kits for carrying out the methods of the invention. Thus, the invention provides a kit for detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising:

(a) means for detecting the presence of blood in the faecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer, and

(b) means for detecting an epigenetic modification in the DNA contained within the faecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer.

As for the corresponding methods, in certain embodiments, the epigenetic modification is detected in at least one gene selected from PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2 and CDO1 and thus the means for detecting an epigenetic modification in the DNA are means for detecting an epigenetic modification in at least one gene selected from PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2 and CDO1 In further embodiments, the epigenetic modification is detected in at least one gene selected from PHACTR3, NDRG4 and FOXE1 and thus the means for detecting an epigenetic

modification in the DNA are means for detecting an epigenetic modification in at least one gene selected from PHACTR3, NDRG4 and FOXE1. The epigenetic modification may be detected in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 , with detection of the epigenetic modification in at least one of the genes providing an indication of a predisposition to, or incidence of, colorectal cancer. Means may be provided for detecting an epigenetic modification in a panel of genes comprising, consisting essentially of or consisting of PHACTR3, NDRG4 and FOXE1, NDRG4 and FOXE1, PHACTR3 and NDRG4, or PHACTR3 and FOXE1. In some embodiments, the means for detecting an epigenetic change in the panel of genes enable the detection to be carried out in a single reaction. Thus the means may permit multiplexing.

In further embodiments, the epigenetic modification is detected in at least one gene selected from LAMA1 and CDO1 and thus the means for detecting an epigenetic modification in the DNA are means for detecting an epigenetic modification in at least one gene selected from LAMA1 and CD01. Suitable primers and probes are set forth in table 1.

In further embodiments, the epigenetic modification is detected in at least one gene selected from GPNMB and MMP2 and thus the means for detecting an epigenetic modification in the DNA are means for detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2. Suitable primers and probes are set forth in table 1.

As for the methods, the epigenetic modification is often methylation. Thus, the means for detecting an epigenetic modification in the DNA contained within the faecal sample may comprise, consist essentially of or consist of primers and/or probes which permit the methylation status of the DNA to be determined directly. In specific embodiments, the primers are selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth in table 1. Suitable primer pairs can be selected from the primers listed in the table based upon the gene or genes of interest. In specific embodiments, the kits incorporate primers selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOXE1), and 7 and 8 (NDRG4). In further specific embodiments, the probes are selected from probes comprising, consisting essentially of or consisting of the nucleotide

sequences set forth in table 1. Suitable probes can be selected from the probes listed in the table based upon the gene or genes of interest. In specific embodiments, the kits incorporate probes selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOX E1), and 9 (NDRG4).

5 The kit may contain any suitable means for allowing detection of blood in a faecal sample. In particular embodiments, the means for detecting the presence of blood in the faecal sample comprise means for detection of haemoglobin in the faecal sample. These may be chromogenic or immunochemical means. Thus, gum guaiac may be incorporated in the kits for chromogenic detection. Alternatively suitable anti-haemoglobin antibodies, as
10 discussed herein, may be incorporated in the kits of the invention. These components may be provided together with suitable buffers etc. as would be readily appreciated by one skilled in the art.

 The kits of the invention may also contain further elements to facilitate sample collection and processing. Thus in certain embodiments, the kit further comprises a sealable
15 vessel for collection of a faecal sample. This vessel may be of a size and construction to permit a subject to directly defecate into the vessel. There may also be provided an additional container to which a removed portion of the sample may be added to allow step (a) to be performed on this separated sample. As mentioned in the methods section, this container may contain or be supplied with an appropriate buffer for protein preservation (to prevent
20 denaturation or degradation of proteins). The kit may also contain suitable means for transfer of the removed portion of the sample to the second container. In view of the fact that the invention may be performed using only a small portion of the (total collected) faecal sample, as discussed herein (for example using as little as approximately 1 g of stool to perform DNA extraction), the second container may be suitably dimensioned to contain such a
25 minimal/portioned sample. The second container may be provided with a suitable collection device, adapted to retrieve and/or deposit an appropriate minimal/portioned sample (as described herein, of approximately 5, 4, 3, 2 or 1g or less). Suitable tubes and spoons for inclusion in such a kit are known in the art and commercially available. The spoons may be measuring spoons and may be integrated into the tubes in certain embodiments. The
30 container may be dimensioned such that a suitable volume of homogenization buffer (as

discussed herein, which discussion applies mutatis mutandis) may be added to the minimal/portioned sample added to the container.

In further embodiments, the kit, or more specifically the means for detecting an epigenetic modification in the DNA, further comprises means for processing a faecal sample.

5 The means for processing a faecal sample comprises a homogenization buffer in certain embodiments. Suitable homogenization buffers are known in the art and commercially available. In related embodiments, the means for processing a faecal sample comprises reagents for extraction/isolation/concentration/purification of DNA. As an example, the QIAamp DNA stool kit available from Qiagen includes suitable components for purification
10 of total DNA from fresh or frozen faecal samples.

In order to permit determination of the methylation status of the DNA, the kits may further comprise a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues. As discussed above, this permits sequence differences
15 to be detected as an indication of the methylation status of the DNA. Any suitable reagent may be employed. Many examples are known in the art and commercially available. In certain embodiments, the reagent comprises a bisulphite reagent. More specifically, the bisulphite reagent may comprise, consist essentially of or consist of sodium bisulphite.

In a related aspect, the invention also provides a kit for any of:

20 (a) detecting a predisposition to, or the incidence of, colorectal cancer in a sample

(b) monitoring treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor

(c) predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor

(d) predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor; or

(e) selecting a suitable treatment regimen for colorectal cancer comprising means for detecting an epigenetic modification in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1.

As discussed herein, detection of the methylation status of this panel of genes provides a sensitive and specific means for detecting colorectal cancer. In certain embodiments, the means for detecting an epigenetic modification in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 comprise primers and/or probes which permit the methylation status of the genes to be determined directly. In specific embodiments, the primers are selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOX E1), and 7 and 8 (NDRG4). In further embodiments, the probes are selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOX E1), and 9 (NDRG4).

Similarly, the invention also provides a kit for any of:

(a) detecting a predisposition to, or the incidence of, colorectal cancer in a sample

(b) predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor

(c) predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor; or (d) selecting a suitable treatment regimen for colorectal cancer comprising means for detecting an epigenetic modification in at least one gene selected from LAMA1 and CDOL

As discussed herein, detection of the methylation status of these genes has been shown herein to provide a sensitive and specific means for detecting colorectal cancer. In

certain embodiments, the means for detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1 comprise primers and/or probes which permit the methylation status of the at least one gene to be determined directly. In specific embodiments, the primers are selected from primers selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 28 and 29 (LAMA1) and 34 and 35 (CDO1). In further embodiments, the probes are selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 30 (LAMA1) and 36 (CD01).

As for the methods, the kits are preferably employed in the context of faecal samples. Thus, the kit further comprises means for processing a faecal sample in certain embodiments. In view of the fact that the invention may be performed using only a small portion of the (total collected) faecal sample, as discussed herein (for example using as little as approximately 1g of stool to perform DNA extraction), the kits may incorporate a container suitably dimensioned to contain such a minimal/portioned sample. The container may be provided with a suitable collection device, adapted to retrieve and/or deposit an appropriate minimal/portioned sample (as described herein, of approximately 5, 4, 3, 2 or 1g or less). Suitable tubes and spoons for inclusion in such a kit are known in the art and commercially available. The spoons may be measuring spoons and may be integrated into the tubes in certain embodiments. The container may be dimensioned such that a suitable volume of homogenization buffer (as discussed herein, which discussion applies mutatis mutandis) may be added to the minimal/portioned sample added to the container.

The invention also provides a kit for any of:

(a) detecting a predisposition to, or the incidence of, colorectal cancer in a sample

(b) predicting the likelihood of successful treatment of colorectal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor

(c) predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor; or (d) selecting a suitable treatment regimen for colorectal cancer comprising means for

detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2 and means for processing a faecal sample.

As discussed herein, detection of the methylation status of these genes has been shown herein to provide a sensitive and specific means for detecting colorectal cancer in the context of faecal samples. In certain embodiments, the means for detecting an epigenetic modification in at least one gene selected from GPNMB and NMP1 comprise primers and/or probes which permit the methylation status of the at least one gene to be determined directly. In specific embodiments, the primers are selected from primers selected from primers comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 22 and 23 (GPNMB) and 31 and 32 (MMP2). In further embodiments, the probes are selected from probes comprising, consisting essentially of or consisting of the nucleotide sequences set forth as SEQ ID Nos: 24 (GPNMB) and 33 (MMP2).

In specific embodiments of these kits of the invention which involve means for processing a faecal sample, the means for processing a faecal sample comprises a sealable vessel for collection of a faecal sample. This vessel may be of a size and construction to permit a subject to directly defecate into the vessel. In view of the fact that the invention may be performed using only a small portion of the (total collected) faecal sample, as discussed herein (for example using as little as approximately 1 g of stool to perform DNA extraction), the kits may incorporate a container suitably dimensioned to contain such a minimal/portioned sample. The container may be provided with a suitable collection device, adapted to retrieve and/or deposit an appropriate minimal/portioned sample (as described herein, of approximately 5, 4, 3, 2 or 1g or less).

Suitable tubes and spoons for inclusion in such a kit are known in the art and commercially available. The spoons may be measuring spoons and may be integrated into the tubes in certain embodiments. The container may be dimensioned such that a suitable volume of homogenization buffer (as discussed herein, which discussion applies mutatis mutandis) may be added to the minimal/portioned sample added to the container.

In further embodiments, the means for processing a faecal sample comprises a homogenization buffer in certain embodiments. Suitable homogenization buffers are known in the art and commercially available. In related embodiments, the means for processing a faecal sample may (further) comprise reagents for
5 extraction/isolation/concentration/purification of DNA from the faecal sample. As an example, the QIAamp DNA stool kit available from Qiagen includes suitable components for purification of total DNA from fresh or frozen faecal samples.

In order to permit determination of the methylation status of the panel of genes, the kits may further comprise a reagent which selectively modifies unmethylated cytosine
10 residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues. As discussed above, this permits sequence differences to be detected as an indication of the methylation status of the DNA. Any suitable reagent may be employed. Many examples are known in the art and commercially available. In certain embodiments, the reagent comprises a bisulphite reagent.
15 More specifically, the bisulphite reagent may comprise, consist essentially of or consist of sodium bisulphite.

EXPERIMENTAL SECTION

Example 1

Sample collection and processing

20 A standardized multicenter screening trial (The Netherlands) was initiated in 2006. In this trial, non symptomatic subjects aged 50 or above are screened with colonoscopy, FOBT and real-time MSP using DNA from stool and blood. In addition, prospectively collected stool samples from multiple centers (Germany and The Netherlands) were used. In these trials, symptomatic patients, attending a Gastroenterology clinic and ultimately diagnosed
25 with CRC, provided a stool sample for use in real-time MSP. From the ongoing trials 111 stool samples were available for the present study. 3 main categories of stool samples were used: 38 samples with no suspicious findings, 10 adenomas and 63 samples from patients covering all stages of CRC. After defecation in a special bucket, an iFOBT samples was

taken (Eiken device) and the patients subsequently added 250 ml of stool homogenization buffer (Amresco, Solon, Ohio, USA) to the sample. Samples were shipped to the laboratory and further processed within 72 hours after defecation. Stool homogenization buffer was added to a ratio 1 :7, and the samples were homogenized and aliquoted in portions of 32 ml.

5 *iFOBT*

The OC-Hemodia 'Eiken' was used to detect faecal occult blood when screening for early identification of colon cancer

DNA extraction from stool

Single aliquots (32 ml containing the equivalent of 4 g of stool) were centrifuged for
10 5 minutes at 2540 rcf at 20°C. The supernatant was retained and centrifuged a second time
(10 minutes at 16500 rcf at 4°C). 22 ml of the supernatant obtained following the second
centrifugation step was incubated with 5 µl Rnase A for 60 minutes at 37°C. Total DNA was
then SodiumAcetate (pH 5.2) - isopropanol precipitated and washed with 70% ethanol. This
"crude DNA" was resuspended in 4 ml 1x TE (pH 7.4). For set A (QIAamp DNA Stool Mini
15 Kit): Add 1.6ml ASL Buffer to 300 µl "crude DNA" and vortex. The samples were
centrifuged to pellet remaining stool particles. 1.4 ml of this supernatant was transferred into
a new microcentrifuge tube. One InhibitEX Tablet was dissolved in each sample before a
1min incubation step to allow inhibitors to adsorb to the InhibitEX matrix. The samples were
then centrifuged to pellet all remaining stool particles and inhibitors bound to InhibitEX
20 matrix. The supernatant was transferred into a new microcentrifuge tube. 25 µl of proteinase
K was added to 600µl of sample supernatant before adding 600 µl of AL Buffer. The tubes
are incubated at 70°C for 10 min. 600 µl of ethanol (96-100%) were added to the lysate, and
mix by vortexing. Carefully apply 3x600 µl of lysate to QIAamp spin columns, centrifuge
and discard the tube containing the filtrate. Carefully open the QIAamp spin column and add
25 500 µl of AW1 Buffer, centrifuge and discard the collection tube containing the filtrate.
Carefully open the QIAamp spin column and add 500 µl of AW2 Buffer, centrifuge discard
the collection tube containing the filtrate. Transfer the QIAamp spin column into a new
microcentrifuge tube, open the QIAamp spin column, add 50 µl of AE Buffer onto the

membrane, incubate for 1 min at room temperature, and then centrifuge to elute DNA. For set B (QIAamp DNA Stool Midi Kit): Add 1.5ml ASL buffer to 2 ml of "Crude DNA". Add 1 inhibitEX Tablet to each sample and vortex IMMEDIATELY and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. Centrifuge sample to pellet stool particles and inhibitors bound to InhibitEX matrix. Collect the supernatant and transfer it into a new 15 ml centrifuge tube, discard the pellet.

Add 150 μ l proteinase K to 2 ml of the supernatant, mix well, add 2.4 ml of AL Buffer and vortex for 15 s. Incubate at 70°C for 10 min. Add 2 ml ethanol (96-100%) to the sample, and mix by inverting the tube 10 times, followed by additional vigorous shaking. In order to ensure efficient binding, it is essential that the sample is mixed thoroughly after addition of ethanol to yield a homogeneous solution.

Carefully transfer 3.3 ml of the sample onto the QIAamp Midi column placed in a 15 ml centrifuge tube and centrifuge at 1850 x g (3000rpm) for 3 min, and discard the filtrate. Place the QIAamp midi column back into the 15 ml centrifuge tube. Load the remainder of the sample onto the QIAamp Midi column, centrifuge again at 1850 x g (3000 rpm) for 3 minutes, and discard the filtrate. Place the QIAamp Midi column back into the 15 ml centrifuge tube, add 2 ml of AW1 Buffer, centrifuge at 4500 x g (5000 rpm) for 1 min, add 2ml of AW2 Buffer to the QIAamp Midi column, centrifuge at 4500 x g (5000 rpm) for 15 min, and discard the filtrate. Place the QIAamp Midi column in a clean 15 ml centrifuge tube, centrifuge for 1 min to dry the column, place the QIAamp Midi column in a clean 15 ml centrifuge tube, and discard the collection tube containing the filtrate.

Add 200 μ l of AE Buffer directly onto the membrane of the QIAamp Midi column, incubate at room temperature for 5 min, and centrifuge at 4500 x g (5000 rpm) for 2 min. Reload the same 200 μ l onto the membrane, incubate at room temperature for 5 min, and centrifuge at 4500 x g (5000 rpm) for 2 min to elute the DNA.

DNA modification

1.5 µg of DNA were subjected to bisulfite modification in 96-wells format on a pipetting robot (Tecan), using the EZ-96DNA Methylation kit (Zymo Research), according to the manufacturer's protocol. Basically, aliquots of 45 µl were mixed with 5 µl of M-Dilution Buffer and incubated at 37°C for 15 minutes shaking at 1100 rpm. Then 100 µl of the diluted CT Conversion Reagent was added and samples were incubated at 70°C for 3 hours, shaking at 1100 rpm in the dark. After conversion, the samples were desalted by incubation on ice for 10 minutes and addition of 400 µl of M-Binding buffer. The samples were loaded on a Zymo-Spin I Column in a collection tube and after centrifugation washed with 200 µl of M-Wash Buffer. 200 µl of M-Desulphonation Buffer was put onto the column and incubated at room temperature for 15 minutes. After centrifugation of the columns, they were washed twice with 200 µl of M-Wash Buffer. Finally, the DNA was washed from the column in 50 µl Tris-HCl 1mM pH8.0 and stored at -80°C, until further processing.

DNA amplification

Real-time MSP was applied on a 7900HT fast real-time PCR system (Applied Biosystems). 5ul or 10ul of the modified DNA was added to a PCR mix (total volume 25 or 50ul) containing buffer (16.6mM (NH₄)₂SO₄, 67 mM Tris (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol), dNTPs (5 mM), forward primer (6 ng), reverse primer (18 ng), molecular beacon (0.16 µM), BSA (0.1 µg), and Jumpstart DNA Taq polymerase (0.4 units; Sigma Aldrich). The primer sequences and molecular beacon sequences used for each of the genes are summarized in table 1. Cycle program used was as follows: 5 minutes 95°C, followed by 45 cycles of 30 seconds 95°C, 30 seconds 57°C (51 °C for APC), and 30 seconds 72°C, followed by 5 minutes 72°C. A standard curve (2x10⁶ - 20 copies) was included to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

RESULTS

Table 1: Primers sequences and beacon sequences (for sample sets A and B)

Gene	Primer or probe	Nucleotide sequence (including labels on probe)	SEQ ID NO:
PHACTR	forward primer	5' - TTATTTTGCAGCGGTTTC - 3'	1
	reverse primer	5' - GAATACTCTAATTCACCGCACT - 3'	2
	beacon	5'-FAM- CGACATGCGGGTTCGGTCGGCGCGGGGCATGTCG-3'-DABCYL	3
FOX1	forward primer	5' - TTTGTTTCGTTTTCGATTGTTTC - 3'	4
	reverse primer	5' - TAACGCTATAAACTCCTACCGC - 3'	5
	beacon	5'-FAM- CGTCTCGTCGGGGTTCGGGCGTATTTTTCAGGTAGGCGAGACG -3'-DABCYL	6
NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'	7
	reverse primer	5' - AATTAAACGAATATAACGCTCGAC - 3'	8
	beacon	5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL	9
GATA4	Forward primer	5'-AGGTTAGTTAGCGTTTTAGGGTC-3'	10
	Reverse primer	5'-ACGACGACGAAACCTCTCG-3'	11
	beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCGACCCAGCATGTCG-DABCYL-3'	12
OSMR	Forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'	13
	Reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'	14
	beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL	15
SYNE1	Forward primer	5'-GTTGGGTTTTTCGTAGTTTTGTAGATCGC-3'	16
	Reverse primer	5'-CTACGCCCAAACCTCGACG-3'	17
	beacon	5'-FAM-CGACATGCCCGGCCCTATCGCCGAAATCGCATGTCG-DABCYL-3'	18
SFRP2	Forward primer	5' - GGGTCGGAGTTTTTCGGAGTTCGCG - 3'	19
	Reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'	20
	beacon	5'-FAM- CGACATGCGGTGTTTCGTTTTTCGCGTTTTAGTCGTCGGGCATGTCG - DABCYL-3'	21
GPNMB	Forward primer	5'-GGTCGTAGTCGTAGTCGGG-3'	22
	Reverse primer	5'-CCGCAAAAACCTAAAACGTAA-3'	23
	beacon	5'-FAM-CGACATGCGGTTTTTTGGGTCGGGGCGCGGCATGTCG-DABCYL-3'	24
SOX17	Forward primer	5'-GAGATGTTTCGAGGGTTGC-3'	25
	Reverse primer	5'-CCGCAATATCACTAAACCGA-3'	26
	beacon	5'-FAM-CGACATGCGTTTCGTGTTTTGGTTTGTGCGGTTTTGGCATGTCG DABCYL-3'	27
LAMA1	Forward primer	5'-TTTTAGATTTATCGAGTGGCG-3'	28
	Reverse primer	5'-CGAACTCACCTCTCTACCGAC-3'	29
	beacon	5'-CGACATGCCAAAAACACGCCCCCGCGCATGTCG-3'	30

MMP2	Forward primer	5'-TTCGGGTTATTAGCGTTTTATC-3'	31
	Reverse primer	5'-ACTCCAACCAAACGACGAA-3'	32
	beacon	5'-FAM-CGACATCGTTGGTTCGGTGCGTGTGGTCGATGTCG-DABCYL-3'	33
CDO1	Forward primer	5'-AATTTGATTGTGTGTGTATCGC-3'	34
	Reverse primer	5'-GAAACGTAAAAATATCGTCGCA-3'	35
	beacon	5'-FAM-CGACATGCGCGATTTCGGATTATTGCGTTGTAGGGCATGTCG-DABCYL-3'	36
TFPI2	Forward primer	5'-GTTTCGTTGGGTAAGGCGTTC-3'	37
	Reverse primer	5'-CATAAAACGAACACCCGAACCG-3'	38
	Beacon	5'-FAM-CGACATGCACCGCGCACCTCCTCCGCCAAGCATGTCG-DABCYL-3'	39

Performance of the individual methylation markers in fecal samples (set A)

Twelve methylation markers (GATA4, GPNMB, TFPI2, FoxE1, SOX17, SYNE1, NDRG4, LAMA, MMP2, PHACTR, OSMR and CDO1) were evaluated in fecal samples.

5 Methylated copies of these genes were quantified in the available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems).

The individual performances of the twelve genes in fecal samples are shown in Table 2. The best performing gene in faecal samples from patients with CRC corresponded to NDRG4 with 61% sensitivity with a corresponding specificity of 100%.

10 **Table 2: Performance of the individual methylation markers in faecal samples set A using a certain cutoff (gene copies).**

Gene	cutoff	Spec (n=21)	Sens (n=23)
GATA4	10	90%	52%
GPNMB	100	100%	39%
TFPI2	100	100%	48%
FoxE1	5	100%	39%
SOX17	10	90%	35%
SYNE1	3	90%	43%
NDRG4	2	100%	61%
LAMA	40	100%	30%
MMP2	10	95%	39%
PHACTR	100	100%	43%
OSMR	1	95%	22%
CDO1	10	95%	13%

Performance of the 3 methylation marker combination in fecal samples set A

The performance of combinations of three methylation markers was also investigated. Methylated copies of the genes were quantified in the available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems). Table 3 shows the results and lists the cut-off (copies) applied. The best combinations had 100% specificity, 70% sensitivity for CRC.

Table 3: Performance of 3 methylation marker combinations in set A (top 3 rows give the cutoffs, empty cells denote that the gene was not used). A sample is scored as positive if any one marker is positive.

NDRG4 (copies)	2	2		2
FOXE1 (copies)	0	0	0	
PHACTR (copies)	100		100	100
Spec (n=21)	100%	100%	100%	100%
Sens (n=23)	70%	70%	52%	65%

Performance of FIT in fecal samples (set A)

FIT was evaluated in the same fecal samples. The performance in fecal samples is shown in Table 4. A specificity of 95% and sensitivity of 78% was obtained.

Table 4: Performance of FIT in fecal samples set A

	FIT
Cutoff (ng/ml)	100
Spec (n=21)	95%
Sens (n=23)	78%

Performance of the 3 methylation marker combination AND FIT in fecal samples set A

The performance of combination of the methylation markers with FIT was investigated. Methylated copies of the genes were quantified in the available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems). Table 5 shows the results and lists the cut-off (copies) applied.

Table 5: Performance of the methylation marker combinations in set A. A sample is scored as positive if any one marker is positive.

Gene	cutoffs (copies)	Methylation marker only		Methylation markers plus FIT (100 ng/ml cutoff)	
		Spec (n=21)	Sens (n=23)	Spec (n=21)	Sens (n=23)
GATA4	10	90%	52%	86%	87%
GPNUMB	100	100%	39%	95%	87%
TFPI2	100	100%	48%	95%	91%
FoxE1	5	100%	39%	95%	87%
SOX17	10	90%	35%	86%	87%
SYNE1	3	90%	43%	86%	91%
NDRG4	2	100%	61%	95%	87%
LAMA	40	100%	30%	95%	83%
MMP2	10	95%	39%	90%	87%

PHACTR	100	100%	43%	95%	87%
OSMR	1	95%	22%	90%	83%
CDO1	10	95%	13%	90%	83%

Performance of the methylation marker combination AND FIT in fecal samples set A

The performance of a combination of the methylation markers with FIT was investigated, the results are shown in table 6.

Table 6: Performance of 3 methylation marker combinations in set A (top 4 rows give the cutoffs, empty cells denote that the gene was not used). A sample is scored as positive if any one marker is positive.

FIT (ng/ml)	100	100	100	100
NDRG4 (copies)	2	2		2
FOXE1 (copies)	0	0	0	
PHACTR (copies)	100		100	100
Spec (n=21)	95%	95%	95%	95%
Sens (n=23)	98%	96%	91%	91%

Performance of methylation markers in fecal samples (set B)

Seven methylation markers (NDRG4, FoxE1, PHACTR, GATA4, OSMR, SYNE1 , SFRP2) were evaluated in fecal samples. Methylated copies of these genes were quantified in the available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems). The individual performances of the 7 genes are shown in Table 7. The best performing gene in fecal samples from patients with CRC corresponded to PHACTR with 68% sensitivity with a corresponding specificity of 100%.

Table 7: Performance of the individual methylation markers in fecal samples set B

Gene	NDRG4	FoxE1	PHACTR	GATA4	OSMR	SYNE1	SFRP2
cutoff	2	0	100	10	1	3	2
Specificity	100%	94%	100%	100%	88%	100%	100%
Sensitivity for advanced adenoma	0%	0%	10%	10%	20%	10%	10%
Sensitivity for CRC	35%	55%	68%	43%	45%	65%	58%

Performance of FIT in fecal samples (set B)

FIT was evaluated in the same fecal samples. The performance in fecal samples is shown in Table 8.

Table 8: Performance of FIT in fecal samples set B

	FIT
Cutoff (ng/ml)	100
Specificity	100%
Sensitivity for advanced adenoma	10%
Sensitivity for CRC	78%

The FIT obtained a sensitivity of 78% and a corresponding specificity of 100%

Performance of the methylation markers and combinations thereof with or without FIT in fecal samples set B

The performance of combination of 3 methylation markers was investigated with or without FIT. Methylated copies of the genes were quantified in the available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems). Table 9 shows the results and lists the cut-off (copies) applied.

Table 9: Performance of the 3 methylation marker combinations in set B

FIT (ng/ml)	100	100	100	100				
NDRG4 (copies)	2	2		2	2	2		2
FOXE1 (copies)	0	0	0		0	0	0	
PHACTR (copies)	100		100	100	100		100	100
Spec (n=17)	94%	94%	94%	100%	94%	94%	94%	100%
Sens adv adenoma (n=10)	20%	20%	20%	20%	10%	0%	10%	10%
Sens CRC (n=40)	95%	88%	95%	95%	70%	58%	70%	68%

Use of FIT as first line screening and methylation as a second line confirmation assay

A simulation was made wherein the FIT test would be used a screening test and whereby a low cutoff of 10 ng/ml would be used to maximize the sensitivity. For samples with more than 10 ng/ml hemoglobin and less than 200 ng/ml the methylation test can be

used to increase the specificity for this subgroup. Samples are scored as positive if the FIT value is higher than 200 ng/ml or if one of the methylation markers is positive.

Table 10 shows the sensitivity and specificity of the FIT test as a first line test, as well as the overall sensitivity and specificity of the combination of FIT as first line and confirmation with "FIT > 200 OR a positive result on a methylation panel consisting of three genes".

Results for panel A when only applying the combination of FIT and methylation markers to samples having more than > 10 and less than 200 ng/ml hemoglobin.

Total number of tested with FIT = 44	Number of patients to be tested with the methylation panel = 5	FOXE1 (copies)	0
		NDRG4 (copies)	0
		PHACTR (copies)	50
		Spec (n=21)	95%
		Sens (n=23)	83%

Results for panel B when only applying the combination of FIT and methylation markers to samples having more than > 10 and less than 200 ng/ml hemoglobin.

Total number of tested with FIT = 67	Number of patients to be tested with the methylation panel = 12	FOXE1 (copies)	0
		NDRG4 (copies)	0
		PHACTR (copies)	50
		Spec (n=17)	94%
		Sens adv adenoma (n=10)	20%
		Sens CRC (n=40)	88%

Example 2

FIT and methylation testing on partial stool samples

In Example 1 homogenates of whole stool sample material were investigated. In this example, the inventor evaluated whether sufficient DNA material could be subtracted from partial stool samples allowing subsequent methylation analysis.

Sample collection and nucleic acid isolation

82 historical stool samples, collected between 2003 and 2008, were available for the present study. The samples were frozen down at the time of collection without addition of stabilizing buffer and stored at -20°C until further use.

5 In this study only a portion of the stool sample ($\approx$ 1g) was investigated for further methylation testing. First, historical samples were thawed at the storage site and partial samples were taken ($\approx$ 1g) using special designed stool tubes with integrated measuring spoons (Sarstedt). Each sample was then weighted and 2 volumes of stabilizing buffer (Diagn
10 MoI Pathol; Volume 14, Number 3, September 2005) were added. Samples were stored at -80°C until the complete sample set was ready for transfer to the processing site.

DNA from these samples was prepared using the QiaAmp DNA stool minikit (Qiagen) following the same procedure as detailed in example 1, sample set A. Adapted volumes were used for starting material and elution: 3 DNA isolations were done per stool sample, processing 250 μ l of homogenized stool material each, and elute in 75 μ l of buffer
15 AE. At the end, eluates of the same sample were pooled and DNA was quantified using the Picogreen® dsDNA quantitation kit (Molecular Probes, #P7589) following the manufacturer's directions.

DNA modification and amplification

20 Samples were subjected to bisulfite modification using a commercially available kit (i.e. Zymo, #D5002) as discussed in example 1. Again, input material and elution volume were slightly adapted for this study: up to 2 μ g of input DNA was used and elution was done in 30 μ l Tris-HCl (1mM, pH 8.0); samples were stored at -80°C, until further processing.

Real-time MSP was performed as set out in example 1. DNA methylation patterns of the PHACTR3 gene were determined by chemical modification of unmethylated but not the
25 methylated cytosines to uracil and subsequent PCR using primers specific for the methylated version, sequence details are provided in Table 1.

Results Methylation status for patients was studied in DNA extracted from partial stool samples derived from historical faecal collections. Average yield of DNA was 7µg (ranging from 0.3 to 27µg, SD 5µg) resulting in sufficient DNA material for subsequent bisulfite modification and real-time MSP procedure. Methylated copies of the PHACTR3 genes were quantified in the available stool portions. Performance of this methylation marker and combination with or without FIT were comparable with the above reported data from the prospectively collected whole stool sample set.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Moreover, all embodiments described herein are considered to be broadly applicable and combinable with any and all other consistent embodiments, as appropriate.

CLAIMS

I claim:

1. 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 the fecal sample to a separate sealable container to produce a removed portion and a remaining portion of the fecal sample;
 - 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; and
 - d) combining the remaining portion of the fecal sample in the sealable collection vessel with a stabilizing buffer, and sealing the sealable collection vessel.
2. The 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.
3. A method of processing a fecal sample, the method comprising:
 - 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;
- b) testing the removed portion of the fecal sample for an amount of a blood protein present in the removed portion;
 - c) extracting nucleic acid from the remaining portion of the fecal sample; and
 - d) testing the nucleic acid for an amount of a human nucleic acid.
4. The method of claim 3, wherein testing the nucleic acid comprises determining expression from a human gene.
5. The 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.
6. The method of claim 5, wherein testing the nucleic acid for the presence of human DNA having an epigenetic modification comprises measuring an amount of a methylated human DNA.
7. The method of claim 5, wherein the epigenetic modification comprises aberrant methylation.
8. The method of claim 7, wherein the aberrant methylation comprises hypermethylation.
9. The method of claim 5, wherein the human DNA having an epigenetic modification comprises a gene and/or a promoter region of a gene.
10. The method of claim 9, wherein the gene is selected from the group consisting of *PHACTR3*, *NDRG4*, *FOXE1*, *GATA4*, *GPNMB*, *TFPI2*, *SOX17*, *SYNE1*, *LAMA*, *MMP2*, *OSMR*, *SFRP2*, and *CDO1*.

11. The method of claim 5, wherein testing the nucleic acid for the presence of human DNA having an epigenetic modification comprises modifying the nucleic acid with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.
12. The method of claim 4, wherein determining expression from the human gene comprises measuring an amount of RNA expressed from the gene.
13. The method of claim 12, wherein measuring an amount of RNA expressed from the gene comprises reverse transcriptase polymerase chain reaction (RT-PCR).
14. The 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.
15. The method of claim 14, wherein testing for the concentration of hemoglobin comprises immunochemical detection of hemoglobin.
16. The method of claim 14, wherein the removed portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the removed portion is at least 5 ng/ml.
17. The method of claim 14, wherein the removed portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the removed portion is at least 10 ng/ml.
18. The method of claim 14, wherein the removed portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the removed portion is at least 20 ng/ml.
19. The method of claim 14, wherein the removed portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the removed portion is at least 50 ng/ml.

20. The method of claim 14, wherein the removed portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the removed portion is at least 200 ng/ml.

ABSTRACT

A method of processing a fecal sample from a human subject comprising removing a portion of a collected fecal sample and adding the removed portion of the sample to a buffer that prevents denaturation or degradation of blood proteins found in the sample, and detecting the presence of human blood in the removed portion of the fecal sample. The method further comprises stabilizing the remaining portion of the fecal sample.

Application Data Sheet 37 CFR 1.76

The Application Data Sheet is part of the provisional or non-provisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.

Inventor Information

of inventors: 1

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Application Information

Customer number 173254 -

Correspondence address ---

Title of invention FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Attorney docket number EXCTD-35239.306

Entity status Regular Undiscounted

Application type Nonprovisional Application under 35 USC 111(a)

Subject matter

Utility

Total number of drawing sheets ---

Suggested figure for publication ---

Filing by reference No

Publication request Normal eighteen-month publication

Representative Information

of representatives: 1

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).

Customer number 173254

Domestic Benefit/National Stage Information

of benefit claims: 5

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c), 386(c), or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior app status Pending
Application number ---
Continuity type Continuation of
Prior app number 15634607
Filing Date 06/27/2017

Prior app status Abandoned
Application number 15634607
Continuity type Continuation of

Patent Owner's Ex. 2004, Page 77

Prior app number **15010436**
Filing Date 01/29/2016

Prior app status Abandoned
Application number **15010436**
Continuity type **Continuation of**
Prior app number **13147570**
Filing Date 03/12/2012

Prior app status Expired
Application number **13147570**
Continuity type **A 371 of international**
Prior app number **PCT/GB2010/000180**
Filing Date 02/03/2010

Prior app status Expired
Application number **PCT/GB2010/000180**
Continuity type **Claims benefit of provisional**
Prior app number **61149581**
Filing Date 02/03/2009

Foreign Priority Information

of foreign priority claims: 1

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the Application Data Sheet constitutes the claim for priority as required by 35 U.S.C. 119 (b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(j)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application number **PCT/GB2010/000180**
Country WO
Filing Date 02/03/2010
Access code ----

Checking this box will cause the application to be examined under the first inventor to file provisions of the AIA.

- ☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization or Opt-Out of Authorization to Permit Access

When this Application Data Sheet is properly signed and filled with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE:

This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

Priority Document Exchange (PDX)

- Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h) (1).
- A.

Search Results from U.S. Application to EPO

- Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).
- B.
- The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

- ☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.
- ☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with the search results from the instant application.

NOTE:

Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Applicant Information

of applicants: 1

The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46.

1. **Exact Sciences Corporation**
5505 Endeavor Lane
Madison, WI 53719
UNITED STATES

Applicant Organization
Applicant type Assignee

Phone: ---
Fax: ---
Email: ---

Assignee Information including Non-Applicant Assignee Information

of assignees: 0

An assignee-applicant identified in the "Applicant" section will appear on the patent application as an applicant.

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

 Data was not provided for this section.

Signature

NOTE:

This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c)

This Application Data Sheet must be signed by a patent practitioner if one or more of the applicants is a juristic entity (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, all joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of all joint inventor-applicants.

See CFR 1.4(d) for the manner of making signatures and certifications.

Signature	First name	Last name	Registration #	Date
/Mary Ann D. Brow/	Mary Ann D.	Brow	42363	09/28/2022
/Mary Ann D. Brow/	Mary Ann D.	Brow	42363	09/28/2022

ELECTRONIC PAYMENT RECEIPT

APPLICATION #
17/936,335

RECEIPT DATE / TIME
09/28/2022 07:52:03 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 # 1513

FILED BY Stephanie Filandrinos

PATENT CENTER # 61015215

AUTHORIZED BY Mary Ann Brow

CUSTOMER # 173254

FILING DATE -

CORRESPONDENCE
ADDRESS -

FIRST NAMED
INVENTOR Joost Louwagie

Payment Information

PAYMENT METHOD
DA / 504302

PAYMENT TRANSACTION ID
E20229RJ54001219

PAYMENT AUTHORIZED BY
Stephanie Filandrinos

FEE CODE	DESCRIPTION	ITEM PRICE(\$)	QUANTITY	ITEM TOTAL(\$)
1830	PROCESSING FEE, EXCEPT IN PROVISIONAL APPLICATIONS	140.00	1	140.00
1817	REQUEST FOR PRIORITIZED EXAMINATION	4200.00	1	4200.00
1111	UTILITY PATENT APPL. SEARCH FEE	700.00	1	700.00
1011	BASIC FILING FEE - UTILITY (PAPER FILING ALSO REQUIRES NON- ELECTRONIC FILING FEE UNDER 1.16(T))	320.00	1	320.00
1311	PATENT APPL. EXAMINATION FEE	800.00	1	800.00

TOTAL	\$6,160.00
AMOUNT:	

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.

Internal Worksheet Only - Do Not Scan

Application Number Receipt Date

☒ New Application ☐ Response To Notice Date Of Response

☐ Jumbo – Large tables are included on the

☐ There are no problems with the CDs in the submission

Items checked below are to be selected in the PALM Pre-Exam CD problem

Omitted Items

☐	Files on compact disc are unreadable:
☐	Disc(s) of either set not readable:
☐	Disc(s) do not contain file names listed:
☐	Amended CD missing unamended files
☐	Missing compact discs

Missing Parts

☐	Disc(s) do not contain file names listed
☐	Discs contains files that are non-ASCII files:
☐	Only one copy of the CD, no duplicate:
☐	Wrong media type, e.g., CD-RW:
☐	The transmittal letter and specification do not list information:
☐	Both disc(s) contain unreadable files:
☐	One disc(s) contain unreadable files:
☐	Compact disc(s) contain viruses, but are still readable:
☐	EFS/Paper Submission:
☐	Table does not maintain data in proper alignment:
☐	Not proper subject matter for CD's:

Informality

☐	No statement in transmittal letter that CD's are the same:
☐	No incorporation by reference statement for the CD's:
☐	CD's not labeled "Copy 1" and "Copy 2":
☐	Deficient CD packaging:
☐	Deficient Labeling on CD's:
☐	The transmittal letter does not list information:
☐	The specification does not list information:
☐	Table less than 51 pages submitted on CD:

File Directory Attachment

☐	Copy of file directory listing is attached and should be mailed to applicant
☐	Copy of file directory listing is not attached
☐	Copy of file directory listing is not attached because it is over one page long

Additional Comments

Doc Code - SEQ.XML

WIPO Type Code -

SLIC Placeholder Sheet for P-ELP (CMS) Content

Application Number: 17936335

Document Date: 09/28/2022

The presence of this form in the CMS record indicates that the following document type was received in electronic format on the date identified above. This content is stored in CMS.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access CMS content via DAV or via the SLIC web page.
- External customers may access CMS content via PAIR/Patent Center Retrieval using the Supplemental Content tab.

Application Number: 17936335

Reviewed by: 97ClN/eHe4jhmn5GMimwpAP70alPRU2kNiDwvOWHBrv8bqgC4kdzMg==

This validation report concerns the sequence listing filed 09-28-2022 and indicates that NO errors were found by the USPTO during validation of the sequence listing. This validation report should not be construed as meaning that the sequence listing complies with all of the requirements of 37 CFR §§ 1.821- 1.825, 37 CFR §§ 1.831- 1.839, WIPO Standard ST.25, or WIPO Standard ST.26

For any sequence listing related questions or concerns, the Applicant is invited to contact the USPTO Sequence Listing Help Desk at 571-272-2510 or SequenceHelpDesk@USPTO.GOV.

Timestamp: [year=2022; month=9; day=29; hr=8; min=29; sec=31; ms=387]

Validated By CRFValidator v 1.5.0

Application No: 17936335 Version No: 1.1

CRF Copy Request from application : Version :

Input Set: /slic/CRF_WORKFLOW/17/936/335/1.1/report_17936335_1_1.xml

Output Set: /slic/CRF_WORKFLOW/17/936/335/1.1/report_17936335_1_1.xml.pdf

Started: 2022-09-28 20:17:06.000

Finished: 2022-09-28 20:17:26.402

Elapsed: 0 hr(s) 0 min(s) 20 sec(s) 402 ms

Total Warnings: 1

Total Errors: 0

No. of SeqIDs Defined: 39

Actual SeqID Count: 39

Error code	Error Description
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number
17/936,335

APPLICATION AS FILED - PART I

(Column 1)		(Column 2)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)		RATE(\$)	FEE(\$)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x =	0
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*				x =	0
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1820

APPLICATION AS AMENDED - PART II

	(Column 1)	(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
		CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)	RATE(\$)	ADDITIONAL FEE(\$)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=		x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x =	
	Application Size Fee (37 CFR 1.16(s))							
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
				TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=		x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x =	
	Application Size Fee (37 CFR 1.16(s))							
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
				TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.



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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
17/936,335	09/28/2022	1797	1820	EXCTD-35239.306	20	2

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

CONFIRMATION NO. 1513

FILING RECEIPT



0000000049645541

Date Mailed: 10/13/2022

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Joost Louwagie, Dornach, SWITZERLAND;

Applicant(s)

Exact Sciences Corporation, Madison, WI;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/634,607 06/27/2017
which is a CON of 15/010,436 01/29/2016 ABN
which is a CON of 13/147,570 03/12/2012 ABN
which is a 371 of PCT/GB2010/000180 02/03/2010

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.)
UNITED KINGDOM PCT/GB2010/000180 02/03/2010 No Access Code Provided

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

Projected Publication Date: 01/19/2023

Non-Publication Request: No

Early Publication Request: No
Title

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Preliminary Class

436

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.



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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/936,335		Joost Louwagie	EXCTD-35239.306

CONFIRMATION NO. 1513

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

WITHDRAWAL NOTICE



OC000000049651334

Date Mailed: 10/13/2022

Letter Regarding a New Notice and/or the Status of the Application

If a new notice or Filing Receipt is enclosed, applicant may disregard the previous notice mailed on 10/13/2022. The time period for reply runs from the mail date of the new notice. Within the time period for reply, applicant is required to file a reply in compliance with the requirements set forth in the new notice to avoid abandonment of the application.

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
<https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html>

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at <http://www.uspto.gov/ebc>.

If the reply is not filed electronically via EFS-Web, the reply must be accompanied by a copy of the new notice.

If the Office previously granted a petition to withdraw the holding of abandonment or a petition to revive under 37 CFR 1.137, the status of the application has been returned to pending status.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/tnguyen/

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number
17/936,335

APPLICATION AS FILED - PART I

(Column 1)		(Column 2)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)		RATE(\$)	FEE(\$)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x =	0
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x =	0
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1820

APPLICATION AS AMENDED - PART II

	(Column 1)	(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
		CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)	RATE(\$)	ADDITIONAL FEE(\$)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=		x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x =	
	Application Size Fee (37 CFR 1.16(s))							
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
				TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=		x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x =	
	Application Size Fee (37 CFR 1.16(s))							
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
				TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
17/936,335	09/28/2022	1797	1820	EXCTD-35239.306	20	1

CONFIRMATION NO. 1513

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

FILING RECEIPT



0000000049651432

Date Mailed: 10/13/2022

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

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Inventor(s)

Joost Louwagie, Dornach, SWITZERLAND;

Applicant(s)

Exact Sciences Corporation, Madison, WI;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/634,607 06/27/2017
which is a CON of 15/010,436 01/29/2016 ABN
which is a CON of 13/147,570 03/12/2012 ABN
which is a 371 of PCT/GB2010/000180 02/03/2010

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 10/12/2022

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/936,335**

Projected Publication Date: 01/19/2023

Non-Publication Request: No

Early Publication Request: No
Title

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Preliminary Class

436

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

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countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

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technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.

Patent Owner's Ex. 2004, Page 110

To: DOCKETING@CASIMIRJONES.COM, Genomic_docketing@cardinal-ip.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 173254

Oct 13, 2022 06:02:01 AM

Dear PAIR Customer:

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 173254 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
17936335	APP.FILE.REC	10/13/2022	EXCTD-35239.306
	M327	10/13/2022	EXCTD-35239.306
	APP.FILE.REC	10/13/2022	EXCTD-35239.306

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If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
17/936,335	09/28/2022	OPAP	6160	EXCTD-35239.306	20	1

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

CONFIRMATION NO. 1513
REPLACEMENT FILING RECEIPT



0000000050730025

Date Mailed: 11/15/2022

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

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Inventor(s)

Joost Louwagie, Dornach, SWITZERLAND;

Applicant(s)

Exact Sciences Corporation, Madison, WI;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/634,607 06/27/2017
which is a CON of 15/010,436 01/29/2016 ABN
which is a CON of 13/147,570 03/12/2012 ABN
which is a 371 of PCT/GB2010/000180 02/03/2010
which claims benefit of 61/149,581 02/03/2009

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 10/12/2022

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/936,335**

Projected Publication Date: 01/19/2023

Non-Publication Request: No

Early Publication Request: No
Title

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306	1513
173254	7590	11/15/2022	EXAMINER	
CASIMIR JONES, S.C.			CENTRAL, DOCKET	
2275 DEMING WAY, SUITE 310				
MIDDLETON, WI 53562				
			ART UNIT	PAPER NUMBER
			OPAP	
			NOTIFICATION DATE	DELIVERY MODE
			11/15/2022	ELECTRONIC

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

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Genomic_docketing@cardinal-ip.com

<i>Decision Granting Request for Prioritized Examination (Track I)</i>	Application No. 17/936,335	Applicant(s) Louwagie, Joost	
	Examiner FIKIRTE A GEREMEW	Art Unit OMBL	AIA (FITF) Status No
1. THE REQUEST FILED <u>28 September 2022</u> IS GRANTED . The above-identified application has met the requirements for prioritized examination A. ☒ for an original nonprovisional application (Track I). B. ☐ for an application undergoing continued examination (RCE). 2. The above-identified application will undergo prioritized examination. The application will be accorded special status throughout its entire course of prosecution until one of the following occurs: A. filing a <u>petition for extension of time</u> to extend the time period for filing a reply; B. filing an <u>amendment to amend the application to contain more than four independent claims, more than thirty total claims</u>, or a multiple dependent claim; C. filing a <u>request for continued examination</u> ; D. filing a notice of appeal; E. filing a request for suspension of action; F. mailing of a notice of allowance; G. mailing of a final Office action; H. completion of examination as defined in 37 CFR 41.102; or I. abandonment of the application. Telephone inquiries with regard to this decision should be directed to FIKIRTE GEREMEW at (703) 756-1930. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.			
/FIKIRTE A GEREMEW/ PROGRAM SUPPORT ASSISTANT, OMBL			

Patent Owner's Ex. 2004, Page 117

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Nov 15, 2022 03:32:59 AM

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2275 DEMING WAY, SUITE 310
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Application	Document	Mailroom Date	Attorney Docket No.
17936335	APP.FILE.REC	11/15/2022	EXCTD-35239.306

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Patent Owner's Ex. 2004, Page 118

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Subject: Private PAIR Correspondence Notification for Customer Number 173254

Nov 16, 2022 03:24:30 AM

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2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
17936335	TRACK1.GRANT	11/15/2022	EXCTD-35239.306

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

CONFIRMATION NO. 1513
NEW OR REVISED PPD NOTICE



NOTICE OF NEW OR REVISED PROJECTED PUBLICATION DATE

The above-identified application has a new or revised projected publication date. The current projected publication date for this application is 01/19/2023. If this is a new projected publication date (there was no previous projected publication date), the application has been cleared by Licensing & Review or a secrecy order has been rescinded and the application is now in the publication queue.

If this is a revised projected publication date (one that is different from a previously communicated projected publication date), the publication date has been revised due to processing delays in the USPTO or the abandonment and subsequent revival of an application. The application is anticipated to be published on a date that is more than six weeks different from the originally-projected publication date.

More detailed publication information is available through the private side of Patent Application Information Retrieval (PAIR) System. The direct link to access PAIR is currently <http://pair.uspto.gov>. Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Questions relating to this Notice should be directed to the Office of Data Management, Application Assistance Unit at (571) 272-4000, or (571) 272-4200, or 1-888-786-0101.

Patent Owner's Ex. 2004, Page 120

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Cc: PAIR_eOfficeAction@uspto.gov
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Nov 18, 2022 03:22:02 AM

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Application	Document	Mailroom Date	Attorney Docket No.
17936335	NTC.PUB.DATE	11/17/2022	EXCTD-35239.306

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306	1513
173254	7590	01/11/2023	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
			01/11/2023	ELECTRONIC

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Patent Owner's Ex. 2004, Page 122 Office Action Summary	Application No. 17/936,335	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 --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 28 SEP 2022.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.

2a) ☐ This action is **FINAL**.

2b) ☒ This action is non-final.

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

4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☒ Claim(s) 1-20 is/are pending in the application.

5a) Of the above claim(s) ____ is/are withdrawn from consideration.

6) ☐ Claim(s) ____ is/are allowed.

7) ☒ Claim(s) 1-20 is/are rejected.

8) ☐ Claim(s) ____ is/are objected to.

9) ☐ Claim(s) ____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☐ The specification is objected to by the Examiner.

11) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

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

Certified copies:

a) ☐ All b) ☐ Some** c) ☐ None of the:

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

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

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

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

3) ☐ Interview Summary (PTO-413)

Paper No(s)/Mail Date ____.

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)

4) ☐ Other: ____.

Paper No(s)/Mail Date 28 SEP 2022.

Detailed Action

- ▶ **Claim(s) 1-20** as presented in the paper(s) filed 28 SEP 2022 is/are 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. 35 U.S.C. 112(b)/ 112 (pre-AIA), second paragraph

Sequence Rules

- ▶ This application complies with the sequence rules and the sequence(s) have been entered by the Scientific and Technical Information Center.

Non-Statutory Obviousness-type Double Patenting

- ▶ The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969). A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b). A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-l.jsp.

► **Claim(s) 1- 3, 5-11 and 14-20** is/are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 82, 84, 87, 89-94 and 96-100 of copending Application No. 15/634,607 – hereinafter “607”. Although the conflicting claims are not identical, they are not patentably distinct from each other.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented., they have been allowed.

► **Claim(s) 4 and 12-13** is/are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 82, 84, 87, 89-94 and 96-100 of copending Application No. 15/634,607 as applied above and further in view of Ohlsson et al. [Br. J. of Cancer 95:218(2006) – hereinafter “Ohlsson”].

Claim 4 is drawn to an embodiment of the method of Claim 1 wherein testing the nucleic acid comprises determining expression from a human gene.

The Claims of the 607' application reasonably suggest a method comprising many but not all of the limitations of Claim 4. For example, Claim 87 teaches analyzing nucleic acid extracted from the remaining portion but fail to teach testing the nucleic acid for expression of a human gene. However, it was well known to analyze human nucleic acids in order to detect CRC in human sample. For example, Ohlsson teaches using quantitative RT-PCR to detect the expression of select genes in order to detect the presence of occult tumour (i.e. CRC) cells in said samples. Accordingly, absent an unexpected result it would have been *prima facie* obvious to the PHOSITA at the time of the invention to modify the method of the claims of the 607' application wherein the nucleic acids tested for are mRNA biomarkers as taught by Ohlsson rather than or in addition to the epigenetic markers of 607'. The PHOSITA would have been motivated by a desire to gather as much data as possible to insure a correct diagnosis.

Claim 12 is drawn to an embodiment of the method of Claim 4 wherein determining expression from the human gene comprises measuring an amount of RNA from the gene.

Claim 13 is drawn to an embodiment of the method of Claim 12 wherein measuring an amount of RNA from the gene comprises RT-PCR.

Ohlsson teaches these limitations, see at least the abstract.

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, Dave Nguyen, can be reached at (571) 272-0731.

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

EXAMINER SEARCH NOTES

19-30 DEC 2022 - ECW

Databases searched: All available via PE2E SEARCH

CAPLUS, Medline and BIOSIS via STNext; and Google Scholar (note the search terms used below)

Reviewed the parent(s), if any, and any search(es) performed therein: see the BIB data sheet
Reviewed and updated the search in 15/634,607, now allowed

Reviewed, the search(es), if any, performed by prior examiners including any international examiners.

Planned Search

Search terms:

All Inventor(s) e.g. Louwagie J?/au

feces or stool or excrement or fecal

devid\$5 or separate

Analyse or analyze or Analysis or test\$4

Methylation or aberrant methylation or hypermethylation\$5 or hypomethylation\$5

CRC or colorectal cancer

gene expression or aberrant gene expression

Notice of References Cited

Application/Control No.
17/936,335

Applicant(s)/Patent Under
Reexamination
Louwagie, Joost

Examiner
Ethan C Whisenant

Art Unit
1634

Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A					
	B					
	C					
	D					
	E					
	F					
	G					
	H					
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	K					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Ohlsson et al., British J. of Cancer 95 218-225 (Year: 2006)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes 	Application/Control No. 17/936,335	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

CPC - Searched*		
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/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	
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<i>Index of Claims</i> 	Application/Control No. 17/936,335	Applicant(s)/Patent Under Reexamination Louwagie, Joost
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✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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APPLICANTS

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FOREIGN APPLICATIONS

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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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Keywords: carcinoembryonic antigen (CEA); CEA cell adhesion molecule (CEACAM); mucin 2 (MUC2); cytokeratin 20 (CK20); matrix metalloproteinase 7 (MMP7).

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; Jantschke *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'-TCTCCTTGAGTTTGGCTTCTAAA-3' and probe 5'-TCTTGAGATAGTCTGAGCCTGTTCCTCA-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
CN86+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).

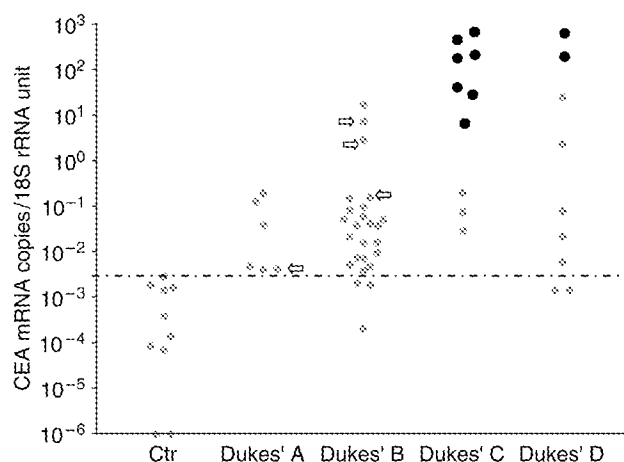


Figure 1 CEA mRNA levels in lymph nodes as determined by real-time qRT-PCR for CEA mRNA and 18S rRNA content in the samples. Each patient is represented by the lymph node with the highest CEA mRNA level (dots). Arrows indicate the four patients with Dukes' Stage A and B tumours, who had died from CRC 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

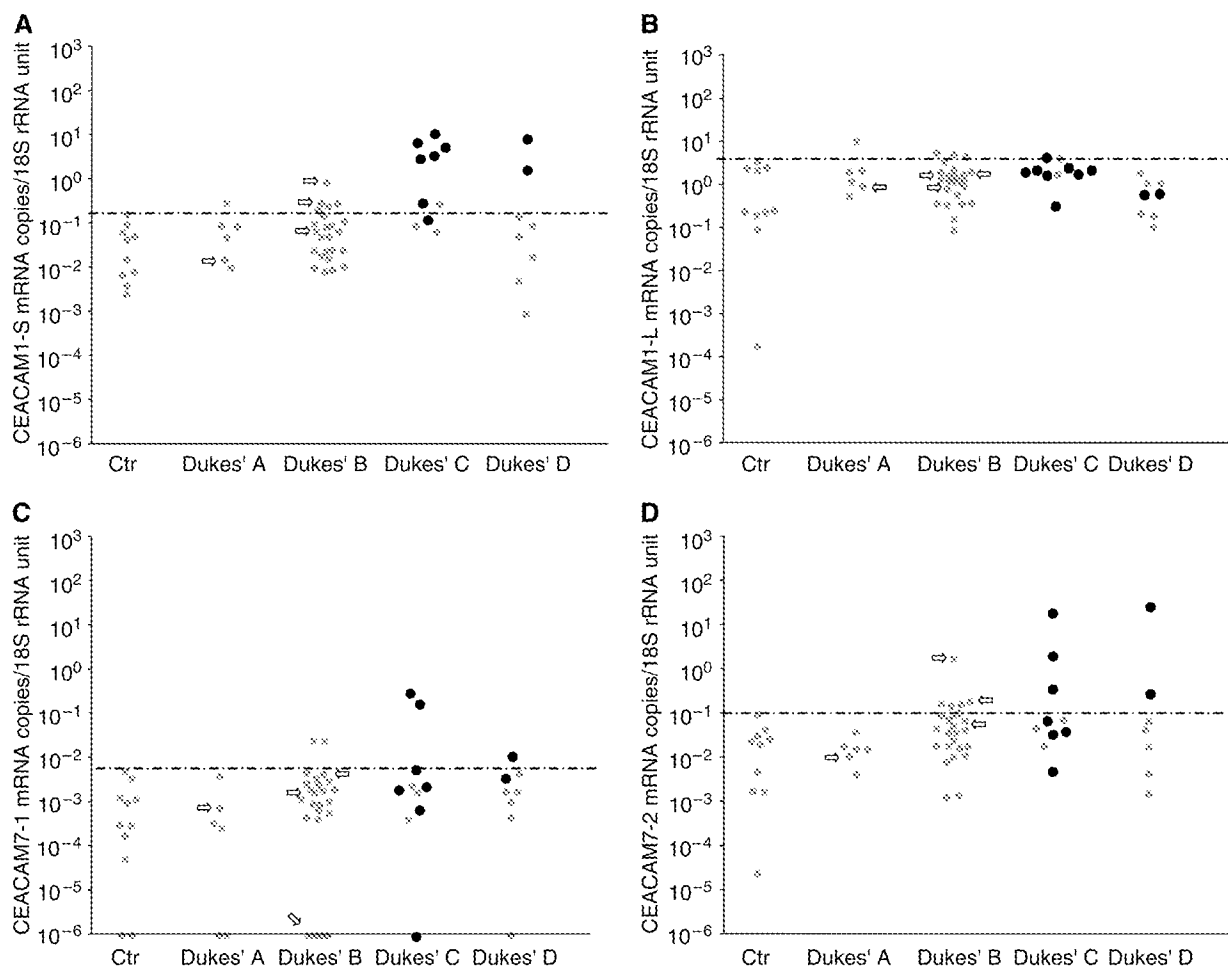


Figure 2 CEACAM1-S (A), CEACAM1-L (B), CEACAM7-1 (C), and CEACAM7-2 (D) mRNA levels in lymph nodes of CRC and control patients. Each patient is represented by the lymph node with the highest level of the respective mRNA species. For explanation of dots and arrows see legend to Figure 1.

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

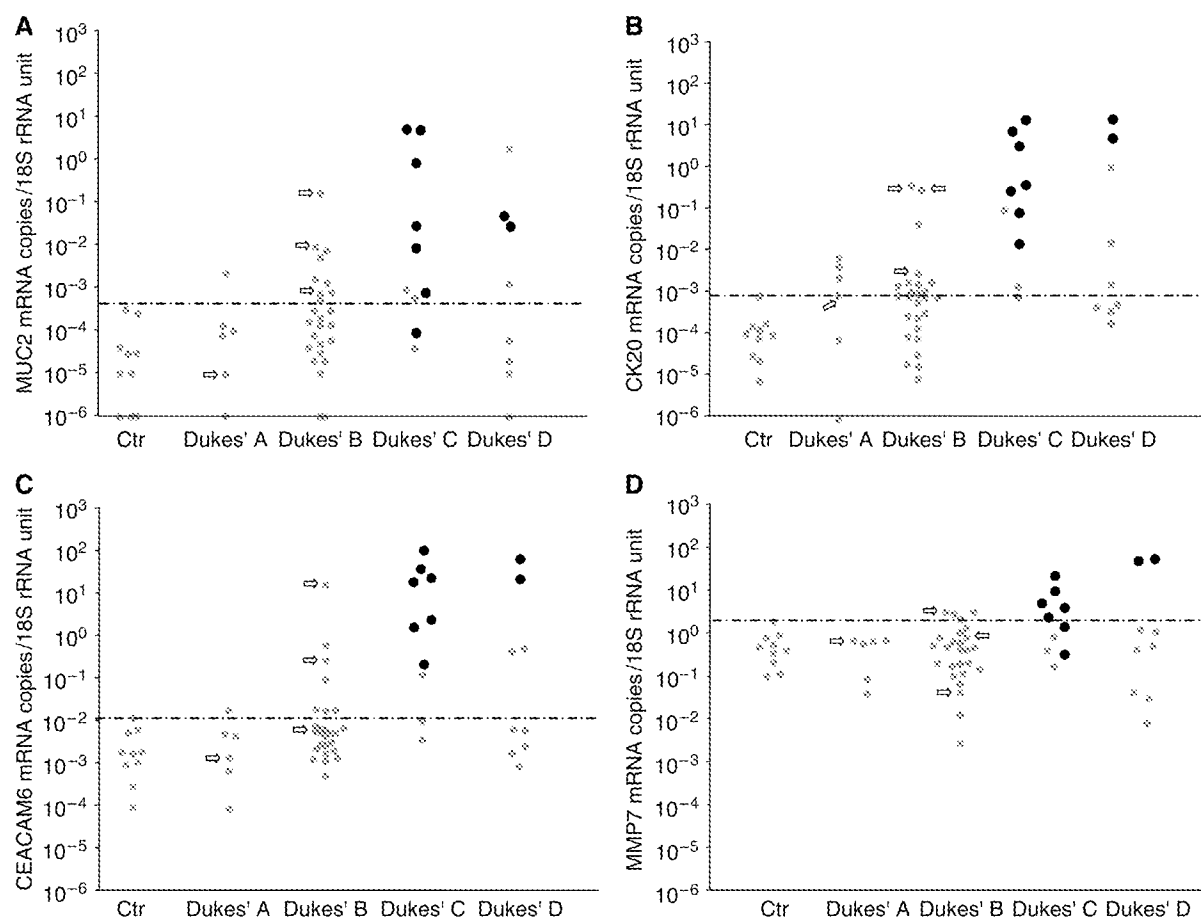


Figure 3 MUC2 (A), CK20 (B), CEACAM6 (C), and MMP7 (D) mRNA levels in lymph nodes of CRC and control patients. Each patient is represented by the lymph node with the highest level of the respective mRNA species. For explanation of dots and arrows see legend to Figure 1.

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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Application	Document	Mailroom Date	Attorney Docket No.
17936335	CTNF	01/11/2023	EXCTD-35239.306
	892	01/11/2023	EXCTD-35239.306
	1449	01/11/2023	EXCTD-35239.306

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Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
17/936,335	09/28/2022

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☒ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above:

173254

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☒ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name

Address

City

State

Zip

Country

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Email

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

EXACT SCIENCES CORPORATION

- ☐ Inventor or Joint Inventor (title not required below)
- ☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)
- ☒ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)
- ☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature

/Jennifer M. Duke/

Date (Optional)

Name

Jennifer M. Duke

Title

Sr. Director, Lead Counsel Intellectual Property

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☒ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

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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.

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #
17/936,335

RECEIPT DATE / TIME
01/23/2023 12:50:32 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 #	1513	FILED BY	Stephanie Filandrinos
PATENT CENTER #	61475561	FILING DATE	09/28/2022
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-01-23_35239- 306__execPoa.pdf	2	Power of Attorney	203 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2023-01-23_35239- 306__execPoa.pdf	90429B3DE65B888EA63D4190D68C9A82C54CF971D8D3BE1F7 DCD31E1FC82FE2BD390D797E0CFE3DCA8A76D8E08E3DFFE 7EAE601E7D3DDBA3650C1958F43F06C

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.



UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

CONFIRMATION NO. 1513
POA ACCEPTANCE LETTER



OC000000053011221

Date Mailed: 01/25/2023

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 01/23/2023.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/qnghuyen/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Joost Louwagie	Confirmation: 1513
Serial No.: 17/936,335	Group No.: 1634
Filed: 09/28/2022	Examiner: Ethan C. Whisenant
Entitled: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

**RESPONSE TO NON-FINAL OFFICE ACTION
MAILED 01/11/2023**

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

This paper is responsive to the Office Action mailed 01/11/2023, with response due by 04/11/2023. Applicant respectfully requests reconsideration in view of the remarks hereinbelow.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: **EXCTD-35239.306**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Remarks are on Page 2.

REMARKS

Claims 1-20 are pending in the present application. In an Office Action mailed 01/11/2023, the Examiner provisionally rejected all claims on the grounds of non-statutory obviousness-type double patenting over claims 82, 84, 87, 89-94 and 96-100 of co-pending Application Ser No. 15/634,607, alone or in further view of Ohlsson *et al.* (Br. J. of Cancer 95:218(2006)).

Solely in the interest of expediting prosecution, Applicant herewith files a terminal disclaimer over Application Ser No. 15/634,607 to obviate the present rejections. Applicant notes that the filing of a terminal disclaimer to obviate a rejection based on non-statutory double patenting is not an admission of the propriety of the rejection.

It is respectfully submitted that all grounds for rejection have been addressed and Applicant's claims should be passed to allowance. 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: January 24, 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 #
17/936,335

RECEIPT DATE / TIME
01/24/2023 04:07:32 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 #	1513	FILED BY	Stephanie Filandrinos
PATENT CENTER #	61484678	FILING DATE	09/28/2022
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 6

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
WO2000050640A1.pdf	33	Foreign Reference	1298 KB
2023-01-24_IDS-SB08_EXCTD-003USCON3.35239_35239.pdf	3	Information Disclosure Statement (IDS) Form (SB08)	161 KB
Warning: This is not a USPTO supplied IDS fillable form. Data in the form cannot be automatically loaded to other USPTO systems.			
WO2005014154A1.pdf	31	Foreign Reference	1267 KB
2023-01-24_TermDisl_EXCTD-	2	Terminal Disclaimer Filed	296 KB

003USCON3.35239_35239-
306.pdf

2023-01-24_ROA_EXCTD- 003USCON3.35239_35239.p df	2	-	109 KB
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2023-01-24_ROA_EXCTD- 003USCON3.35239_35239- A....pdf	(1-1)	1	Amendment/Request for Reconsideration-After Non- Final Rejection	90 KB
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2023-01-24_ROA_EXCTD- 003USCON3.35239_35239- REM.pdf	(2-2)	1	Applicant Arguments/Remarks Made in an Amendment	105 KB
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Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
WO2000050640A1.pdf	3DAA5D5C9CC7BFA6A290F2039905086857281DC6F61B6FE6A 535DFE23693D254A47F8F50A92ABA4F67C1CBFB31726459B0 F584211208893E8695681A0B6E8738
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WO2005014154A1.pdf	4B1DF29ED26D4CC3058416C4362B64078ABBF42DE5F58CFF D7E661EC34B459B324D7E831F702FD7D241AD48CF3A159914 FD8DEE6DC57F79554A188D53785C9A1
2023-01-24_TermDisl_EXCTD- 003USCON3.35239_35239- 306.pdf	CABC68105D9E86102F54C32C1330B04BF2D35A13D782F14B4 A4C5036275892AAE59074EEB469FEA2809469A6D2B858EF579 7D19DCA2B69F9020C1D5A75A5A174
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Patent Owner's Ex. 2004, Page 156

2023-01-24_ROA_EXCTD- 003USCON3.35239_35239- A....pdf	94024F8132ABAD607D90D10E86BED2CEC8C12F8DF03446533 DA8301CA646C4BB941C8E2C533DE267AEFF88B9207C3BF61 E9A8EABCB4E14DB6747D425249A1790
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2023-01-24_ROA_EXCTD- 003USCON3.35239_35239- REM.pdf	F672823D81DABED887C11121E7C97C7781319DC00DC1BBC1 C6CDDF8E2CD3C52D259117AA3F031A6BDAEBEEFFFAD0A49 64D401FEEBD4F929778C89D1505F94731
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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.

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REJECTION OVER A PENDING "REFERENCE" APPLICATION**

Docket Number (Optional)

EXCTD-35239.306

In re Application of: Joost Louwagie

Application No.: 17/936,335

Filed: 09/28/2022

For: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

The applicant, EXACT SCIENCES CORPORATION, owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending **reference** Application Number 15/634,607, filed, 06/27/2017, as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the **reference** application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said **reference** application, "as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application," in the event that: any such patent granted on the pending **reference** application expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.

I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. ☒ The undersigned is an attorney or agent of record. Reg. No. 42363

/Mary Ann D. Brow/

Signature

24 January 2023

Date

Mary Ann D. Brow

Typed or printed name

Attorney

Title

608-662-1277

Telephone Number

- ☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included.

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

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Routine uses of the information in this record may include disclosure to: 1) law enforcement, in the event that the system of records indicates a violation or potential violation of law; 2) a Federal, state, local, or international agency, in response to its request; 3) a contractor of the USPTO having need for the information in order to perform a contract; 4) the Department of Justice for determination of whether the Freedom of Information Act (FOIA) requires disclosure of the record; 5) a Member of Congress submitting a request involving an individual to whom the record pertains, when the individual has requested the Member's assistance with respect to the subject matter of the record; 6) a court, magistrate, or administrative tribunal, in the course of presenting evidence, including disclosures to opposing counsel in the course of settlement negotiations; 7) the Administrator, General Services Administration (GSA), or their designee, during an inspection of records conducted by GSA under authority of 44 U.S.C. 2904 and 2906, in accordance with the GSA regulations and any other relevant (i.e., GSA or Commerce) directive, where such disclosure shall not be used to make determinations about individuals; 8) 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)); 9) the Office of Personnel Management (OPM) for personnel research purposes; and 9) the Office of Management and Budget (OMB) for legislative coordination and clearance.

If you do not furnish the information requested on this form, the USPTO may not be able to process and/or examine your submission, which may result in termination of proceedings, abandonment of the application, and/or expiration of the patent.

Additional Uses

Additional USPTO uses of the information in this record may include disclosure to: 1) the International Bureau of the World Intellectual Property Organization, if the record is related to an international application filed under the Patent Cooperation Treaty; 2) the public i) after publication of the application pursuant to 35 U.S.C. 122(b), ii) after issuance of a patent pursuant to 35 U.S.C. 151, iii) 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, or iv) without publication of the application or patent under the specific circumstances provided for by 37 CFR 1.14(a)(1)(v)-(vii); and/or 3) the National Archives and Records Administration, for inspection of records.



ELECTRONIC PAYMENT RECEIPT

APPLICATION #
17/936,335

RECEIPT DATE / TIME
01/24/2023 04:07:32 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 # 1513

FILED BY Stephanie Filandrinos

PATENT CENTER # 61484678

AUTHORIZED BY Mary Ann Brow

CUSTOMER # 173254

FILING DATE 09/28/2022

CORRESPONDENCE
ADDRESS -

FIRST NAMED
INVENTOR Joost Louwagie

Payment Information

PAYMENT METHOD
CARD / 0638

PAYMENT TRANSACTION ID
E20231NG09062020

PAYMENT AUTHORIZED BY
Stephanie Filandrinos

FEE CODE	DESCRIPTION	ITEM PRICE(\$)	QUANTITY	ITEM TOTAL(\$)
1806	SUBMISSION OF AN INFORMATION DISCLOSURE STATEMENT	260.00	1	260.00
1814	STATUTORY DISCLAIMER, INCLUDING TERMINAL DISCLAIMER	170.00	1	170.00
TOTAL AMOUNT:				\$430.00

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.

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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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	17936335
				Filing Date	09/28/2022
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT
Sheet	1	of	2	Attorney Docket Number	EXCTD-35239.306

U.S. PATENTS					
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		20020096469	2002-07-25	FAULKNER	
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		WO 2000/050640	2000-08-31	EXACT LABORATORIES, INC.		
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(54) Title: METHODS FOR PRESERVING DNA INTEGRITY (57) Abstract Methods for extracting DNA from a biological sample that result in a higher yield of target DNA than conventional methods. More particularly, methods for extracting DNA include exposing the biological sample to inhibitors of DNA degradation.		

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Methods for Preserving DNA Integrity

Cross-Reference to Related Application

The present application claims priority to and the benefit of U.S. provisional patent application serial number 60/122,177, filed February 25, 1999, the entire disclosure of which is incorporated herein by reference.

Technical Field

5 The invention provides methods for deoxyribonucleic acid ("DNA") extraction from a biological sample. More particularly, the invention relates to methods for high yield DNA extraction from a heterogeneous biological sample by inhibiting DNA degradation.

Background of the Invention

DNA is a relatively stable molecule that is routinely isolated from biological samples.

10 Recently, many diseases involving instabilities (*e.g.*, mutations) in genomic DNA have been characterized. Also, many pathogens have been identified by the presence or absence of a particular DNA in a biological sample. Many diseases, such as cancer, are optimally detected early in their progression. In order for early detection to be effective, relatively low levels of DNA which are indicative of cancer must be detected against a high background of other DNA

15 (*e.g.*, normal human DNA, bacterial DNA, etc.). This type of detection is technically difficult and typically results in low sensitivity of detection. Moreover, in certain complex specimens, including stool, what little species-specific DNA exists, is rapidly degraded, making efficient sequence-specific detection even more difficult. Thus, a need exists for methods to retain integrity of DNA in a sample, especially in samples in which the DNA to be detected is in low

20 proportion relative to other DNA in the sample and is degraded quickly.

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Summary of the Invention

The present invention provides methods for preserving the integrity of DNA in a sample. In a preferred embodiment, methods of the invention prevent enzyme-mediated DNA degradation. Preservation of DNA integrity facilitates isolation and detection of DNA.

5 Methods of the invention are especially useful for extracting or detecting DNA in a biological specimen, especially one that contains low levels of relevant DNA. A good example of a specimen that contains lower-levels of relevant DNA is stool. Typical human stool contains only small amounts of intact human DNA. Most of the human DNA in stool from a healthy individual is presumably from exfoliated epithelial cells, and has undergone apoptotic
10 degradation. As the forming stool passes through the colon, colonic epithelial cells are sloughed onto the stool as part of the cellular turnover that occurs in the colon. Stool also contains sloughed cells from other luminal sources (*e.g.*, lung, stomach, esophagus, *etc.*) Sloughed cells typically have undergone or are undergoing apoptosis, leaving cellular DNA in small fragments. Enzymes, such as deoxyribonuclease ("DNase") and Micrococcal nuclease contribute to the
15 degradation of any intact human DNA that remains. Prior art methods, while using DNase inhibitors, have failed to achieve significant yields of intact, species-specific DNA from stool. Therefore, such methods failed to consider optimization of inhibition of DNA degradation. Methods of the invention are based on the realization that optimal inhibition of DNA degrading enzyme(s) effectively preserves DNA, especially large, diagnostically-relevant DNA fragments
20 that are present in a sample.

In one aspect, the invention comprises inhibiting nucleic acid degradation in a sample and optionally extracting a target DNA with, for example, a phenol-chloroform extraction. Preferably, the inhibition of nucleic acid degradation is sufficient to produce a critical number of

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molecules of analyzable DNA. In one embodiment, methods of the invention comprise inhibiting an enzyme capable of DNA degradation in a stool sample. In a preferred embodiment, methods of the invention comprise exposing a stool sample to an ion chelator, such as a divalent ion chelator. Ion chelators, in certain embodiments inhibit DNase. Examples of preferred inhibitors include ethylenediaminetetraacetic acid ("EDTA"). Additional preferred methods of the invention comprise exposing a stool sample to a Micrococcal nuclease inhibitor, such as EGTA, also a divalent ion chelator. Inhibitors of DNA degradation may be used either alone or in combination to achieve optimal levels of DNA preservation.

Methods of the invention are practiced using any inhibitor of DNA degradation. The amount of inhibitor varies depending on the inhibitor that is used. However, an inhibitor must be used in an amount that preserves significant levels of DNA in the sample for subsequent analysis. Methods for determining sufficient levels of DNA are presented below. Such methods allow the skilled artisan to practice the invention with specificity regardless of the inhibitor used. According to preferred methods, an amount of inhibitor is used that preserves sufficient DNA in the sample for detection of a target DNA within a desired level of statistical confidence. Using methods described herein, the skilled artisan can determine an appropriate amount of any inhibitor for use in methods of the invention. The use of various specific inhibitors is exemplified below.

In another preferred embodiment, methods of the invention comprise obtaining a representative (circumferential or cross-sectional) stool sample, exposing the sample or a portion thereof to a DNase inhibitor, and isolating DNA from the sample. One preferred DNase inhibitor is EDTA. Preferred amounts of EDTA are from about 0.042 g per gram of stool to about 0.782 g per gram of stool and especially from about 0.250 g per gram of stool to about

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0.521 g per gram of stool. DNA may be extracted, for example, by a phenol-chloroform extraction. After extraction, the DNA may be analyzed by methods known in the art. For example, U.S. Patent No. 5,830,665 and U.S. Patent No. 5,670,325, which are incorporated by reference herein, disclose methods for analyzing DNA which has been extracted from a stool sample.

Methods of the invention are useful in any sample in which inhibition of DNA degradation is desired. For example, methods of the invention are especially effective in samples comprising exfoliated cells, especially exfoliated epithelial cells. The DNA contained in such samples typically degrades rapidly, making analysis of a particular DNA, especially one that exists in low proportion within the sample, difficult. For example, such samples include stool, sputum, urine, pus, and colostrum. Methods of the invention include inhibiting DNA degradation in such samples, thus preserving a sufficient amount of DNA for specific, sensitive detection. Any of the features described above, such as DNA degradation inhibitors or amounts of inhibitors that are used, can be useful in samples containing exfoliated cells.

Brief Description of the Drawings

FIG. 1 shows a flow chart describing one aspect of the invention.

FIG. 2 shows a separation gel of DNA isolated from several different homogenized stool supernatants that contained various concentrations of EDTA.

FIG. 3 shows a separation gel of DNA isolated from homogenized stool supernatant that contained various concentrations of EDTA followed by capture and amplification.

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FIG. 4 shows separation gel of DNA isolated from homogenized stool supernatant that contained various concentrations EDTA followed by capture, addition of more DNA, and amplification.

FIG. 5 shows a set of curves produced by regression analysis of the data obtained using the model as described for, for example, Tables 1 and 2.

Detailed Description of the Invention

I. Introduction

The present invention provides methods for increased yield of DNA in a biological sample by preserving the integrity of DNA in the sample. Such methods are especially useful when the DNA of interest ("the target DNA") is present in the sample at a low frequency, or is rapidly degraded. More particularly, methods of the invention include, for example, inhibiting enzymes that degrade DNA.

Prior to the present invention, those skilled in the art have not been concerned with preventing DNA degradation prior to extraction from a sample. Typically, either the DNA of interest is present in samples in relatively large quantities (*e.g.*, tumor cells, blood), or methods are directed toward increasing sensitivity to low-frequency DNA, and not to preserving its integrity. However, especially in the case of low-frequency DNA in a heterogeneous sample (*e.g.*, a sample having cells and/or cellular debris from multiple cell types and/or organisms), methods for increasing sensitivity to DNA have not been entirely successful. Methods of the invention provide a new approach by preserving the integrity of DNA. Methods of the invention increase the likelihood of detecting a specific target DNA, because such methods make more intact target DNA available in the sample.

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Referring to Figure 1, one generalized method of the invention involves obtaining a sample (step 2) and exposing it to a DNA degradation inhibitor (step 4). Once the sample has been exposed to the DNA degradation inhibitor, target DNA is extracted (step 6). The presence or absence of this extracted target DNA is then detected (step 8).

5 In heterogeneous samples, such as stool, endogenous human DNases and/or bacterial nucleases degrade DNA. Examples of nucleases include DNases, such as deoxyribonuclease I ("DNase I") and Micrococcal nuclease. DNase and Micrococcal nuclease both require a divalent cation to function optimally. For DNase, suitable ions include Mn^{+2} and Mg^{+2} . For Micrococcal nuclease, Ca^{+2} is a suitable ion. Ion chelators, and particularly divalent ion chelators, are capable
10 of inhibiting nucleases. Ion chelators remove ions from association with the nuclease, thus inhibiting the nuclease's function. For example, EDTA or EGTA used in optimal amounts are useful ion chelators for use in the present invention.

Other compounds that inactivate, interfere with, or slow enzyme-mediated degradation of DNA are useful. For example, ligands and/or antibodies which compete for or interfere with the
15 active site of DNA degrading enzymes, which inactivate those enzymes, and/or which block messenger systems that control DNA degrading enzymes are useful in the practice of the invention. Phenol-chloroform extraction components, at higher concentrations than those typically used during extraction, also are capable of inhibiting nucleases by separation and denaturation. For example, phenol denatures DNA degradation enzymes, and is used in methods
20 of the invention to preserve DNA integrity. Also, proteinases which degrade and/or denature DNA degrading enzymes are useful.

Methods of the invention comprise the use of optimal amounts of DNA degradation inhibitors in order to preserve high-integrity DNA sufficient for diagnostic screening. In

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heterogeneous samples, such as stool, target DNA (*e.g.*, mutated DNA or reductions in enumerated wild type DNA that are indicative of a mutation) is present in low amounts. An optimal amount of a DNA degradation inhibitor is an amount that results in a measurable improvement in the quantity of DNA available in the sample. Thus, the skilled artisan can empirically determine optimal amounts of DNA degradation inhibitors for use in methods of the invention by using inhibitor amounts necessary to preserve a diagnostically-relevant fraction of high-integrity target DNA. A method for determining diagnostically-relevant DNA amounts is presented below.

Amplification of DNA, and other stochastic processes, performed on heterogeneous samples may actually contribute to the inability to measure low-frequency DNA. For example, a typical cancer-associated (mutant) DNA in the early stages of oncogenesis represents about 1% of the DNA in a heterogeneous sample (*e.g.*, stool). If DNA in the sample is amplified at 30% PCR efficiency, any particular DNA has only a 30% chance of being amplified in any round of PCR. Thus, if a mutant DNA initially present as 1% of a sample is not amplified in the first round, the mutant DNA will represent only about 0.7% of the DNA in the sample after round 1. If no mutant is amplified in the first two rounds (0.7×0.7 , or a 49% probability), the mutant DNA will represent only about 0.6% of the DNA in the sample going into round three of the PCR. If the post-amplification assay used to detect the mutant has a sensitivity of no more than 0.5% for the mutant, it may not be possible to reliably detect the presence of the mutant DNA. Thus, the detection method itself may actually contribute to difficulties in detecting low-frequency DNA, especially if sufficient amounts of intact DNA are not present in a sample. Thus, one means for determining an appropriate amount of inhibitor to use in methods of the invention is to determine the minimum amount of intact DNA that must be present in a sample to

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avoid the stochastic effects described above, and then to use sufficient inhibitor to produce at least the minimum number of DNA molecules in the sample. Methods for calculating the minimum number of DNA molecules necessary to overcome the effects of stochastic processes, such as PCR, are presented below.

5 A model useful to generate sufficient DNA molecules for accurate measurement operates by iterating stochastic processes over a number of rounds of PCR. In the context of molecular disease diagnostics, the model dictates the number of molecules that must be presented to the PCR in order to reliably ensure amplification of desired target DNA. The model incorporates a preset PCR efficiency (established to meet separate specificity requirements), and a preset ratio
10 of mutant DNA to total DNA in the sample to be analyzed (which is a property of the disease to be detected and the nature of the sample). Based upon those input values, the model predicts the number of molecules that must be presented to the PCR in order to ensure, within a predefined level of statistical confidence, that a low-frequency (target) molecule will be amplified and detected. Once the number of molecules is determined, the skilled artisan can determine the
15 sample size to be used (*e.g.*, the weight, volume, etc.), depending on the characteristics of the sample (*e.g.*, its source, molecular makeup, etc.). The model dictates the number of molecules that must be presented to the PCR in order to reliably ensure amplification and detection.

 The exemplary model simulates selection of DNA for amplification through several rounds of PCR. For purposes of the model, a sample is chosen that contains a ratio of mutant-to-
20 total DNA of 1:100, which is assumed to lie at the clinical threshold for disease. For example, in colorectal cancer 1% of the human DNA in a specimen (*e.g.*, stool) is mutated (*i.e.*, has a deletion, substitution, rearrangement, inversion, or other sequence that is different than a corresponding wild-type sequence). Over a large number of PCR rounds, both the mutant and

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wild-type molecules will be selected (*i.e.*, amplified) according to their ratio in the specimen (here, nominally 1 in 100), assuming there are any abnormal molecules in the sample. However, in any one round, the number of each species that is amplified is determined according to a Poisson distribution. Over many rounds, the process is subject to stochastic errors that reduce the ability to detect low-frequency mutant DNA. However, the earlier rounds of PCR (principally, the first two rounds) are proportionately more important when a low-frequency species is to be detected, and any rounds after round 10 are virtually unimportant. Thus, the model determines the combined probability of (1) sufficient mutant molecules being presented to the PCR, and (2) the effects of stochastic amplification on those molecules so that at the output of the PCR there will be a sufficient number of molecules and a sufficient ratio of mutant to total molecules to assure reliable detection.

The model used to run the number of molecules necessary at the first round of PCR was generated as a "Monte Carlo" simulation of a thousand experiments, each experiment consisting of 10 cycles of PCR operating on each molecule in the sample. The simulation analyzed (1) taking a sample from the specimen; and (2) each round of PCR iteratively to determine whether, for each round, a mutant DNA if present in the sample was amplified. Upon completion of the iterative sampling, the model determined the percent of rounds in which a mutant strand was amplified, the percent of mutants exceeding a predetermined threshold for detection (in this example 0.5% based upon the mutant:total ratio of 1%), the coefficient of variation (CV) for stochastic sampling in each round alone, and the coefficient of variance for stochastic sampling and PCR in combination.

Stochastic noise is created in PCR if the PCR efficiency is anything other than 0% or 100% (these two cases represent either there is no amplification at all or perfect fidelity of

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specific amplification). The noise, or background, signal level in a PCR that is between 0% and 100% varies with the efficiency of the PCR. The standard deviation of stochastic noise, S , in a PCR is given by the equation, $S = \sqrt{npq}$, where n is the number of molecules in the sample, p is the efficiency of PCR, and q is $1-p$. Table 1 presents results obtained for iterative samplings
 5 with PCR efficiency set at 100% and 20%, and a mutant:total ratio of 0.5%.

Table 1 represents output from the model in 12 experiments conducted under various conditions. The first row shows the nominal number of molecules entering the first round of PCR (i.e., the total number of molecules available for amplification). The second row shows the percent of molecules (DNA) in the biological specimen that is expected to be mutant. For
 10 colorectal cancer indicia in DNA recovered from stool, the threshold for clinical relevance in the detection of early stage cancer is 1%. That is, 1% of the DNA in a sample derived from a heterogeneous specimen (e.g., stool) contains a mutation associated with colorectal cancer. The 6th row is the threshold of detection of the assay used to measure PCR product after completion of PCR. That number is significant, as will be seen below, because sufficient mutant DNA must
 15 be produced by PCR to be detectable over aberrant signal from wild-type and random background noise. Under the heading "Outputs", the first line provides the likelihood that at least one mutant molecule is presented to the first round of PCR. The second line under the Output heading provides the likelihood of detection of mutants (after PCR) above the predetermined threshold for detection. For example, in experiment 4, the results indicate that in
 20 87.9% of experiments run under the conditions specified for experiment 4, the number of mutants will exceed the threshold number for detection. Finally, the last two rows provide the coefficient of variation for sampling, and for the combination of sampling and PCR.

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TABLE 1

	100% Efficiency PCR						20% Efficiency PCR					
Inputs	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	Exp 7	Exp 8	Exp 9	Exp 10	Exp 11	Exp 12
Nominal number of Molecules going into PCR	50	100	200	500	1,000	10,000	50	100	200	500	1,000	10,000
Percent of molecules that are mutant	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%
Number of PCR Rounds	10	10	10	10	10	10	10	10	10	10	10	10
Efficiency of PCR per round	100%	100%	100%	100%	100%	100%	20%	20%	20%	20%	20%	20%
Number of experiments modeled	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
M:W ratio which can be reliably detected by assay	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%
Outputs												
% of Experiments each with the number of mutant cells > 0	39.30%	64.40%	87.30%	99.40%	100%	100%	39.10%	63.50%	86.10%	99.60%	100%	100%
% of Experiments each with number of mutant cells exceeding threshold	39.30%	64.40%	87.30%	87.90%	97.10%	100%	36.20%	52.40%	63.30%	63.30%	91.60%	100%
Stochastic Sampling CV	143.20%	100%	70%	44.10%	32.10%	9.90%	144.60%	101.80%	72.00%	44.20%	32.00%	10%
Stochastic Sampling and Stochastic PCR CV	143.20%	100%	70%	44.10%	32.10%	9.90%	179.70%	123.10%	92.10%	56.40%	40.30%	12.70%

As shown in Table 1, even at 100% PCR efficiency, mutant DNA is detected in only 97.1% of the samples when 1000 input molecules are used (*i.e.*, 1000 DNA molecules are available for priming at the initial PCR cycle), even though 100% of the DNA is amplified in any given round of PCR. When 10,000 molecules are presented, it is virtually certain that the mutant DNA will be amplified and detected, as shown in the results for experiment 6 in Table 1.

Stochastic errors due to variation in the number of input molecules become less significant at about 500 input molecules and higher (*i.e.*, the CV for stochastic variations is about the same regardless of whether PCR efficiency is 20% or 100%). At lower PCR efficiency (20% in Table 1), the model shows that introducing 50, 100, 200, 500, or even 1000 molecules into the PCR does not assure either amplification or detection. As shown in experiment 12, introducing 10,000 molecules results in amplification of the mutant target, and a high likelihood of its subsequent detection. Thus, even with 100% efficient PCR, significant false negative events occur when input molecules fall below 500.

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The foregoing analysis shows that there is a unique range for the number of molecules that must be presented to a PCR in order to achieve amplification of a low-frequency DNA, and to allow its detection. That range is a function of the PCR efficiency, and the percentage of low-frequency (mutant) DNA in the sample, and the detection threshold. The aforementioned model
5 was developed and run in Visual Basic for Applications code (Microsoft, Office 97) to simulate a PCR as described above. The statistical confidence level within which results were measured was held constant at approximately 99%. Only the PCR efficiency and percent mutant DNA were varied. As discussed above, the model iteratively samples DNA in a "Monte Carlo" simulation over a thousand experiments, each experiment consisting of 10 rounds of PCR. The
10 results are shown below in Table 2.

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TABLE 2

PCR Efficiency	Number of molecules needed			
	1% Mutant	2% Mutant	5% Mutant	10% Mutant
10%	3,000			
20%	2,500			
50%	2,200			
100%	1,600			
10%		1,500		
20%		1,200		
50%		1,000		
100%		800		
10%			500	
20%			450	
50%			400	
100%			300	
10%				225
20%				200
50%				150
100%				125

Regression of the data obtained using the model as described above produced the set of curves set forth below in Figure 5.

5 Using Figure 5, the optimal number of molecules to be presented to the PCR is determined by selecting a PCR efficiency (or determining the efficiency by empirical means), and selecting a percentage of the sample suspected to be mutant DNA associated with disease. This, in turn, dictates a threshold of detection. Not all detection strategies have similar underlying detection thresholds, so an appropriate technology must be selected. The percentage

10 mutant DNA may be determined by clinical considerations as outlined above for colorectal cancer.

One may determine the PCR efficiency and percent expected mutant in order to maximize the probability of obtaining amplified, detectable mutant DNA. For example, one may select N, the number of input molecules from the "1%" curve in Figure 5, when 5% of the

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sample is expected to be mutant DNA in order to increase the confidence of the assay result.

This model, and particularly Figure 5, are useful when determining optimal concentrations of a DNA degradation inhibitor. If PCR is used to analyze DNA after the DNA sample is exposed to a DNA inhibitor, Figure 5, will indicate how many molecules of target DNA need to be preserved in order to have sufficient analyzable DNA. Thus, the optimal amount of any DNA degradation inhibitor may be determined as that amount, or range of amounts, of inhibitor that produce a sufficient number of analyzable DNA according to Figure 5. Of course, this modeling system may be applied to DNA detection techniques other than PCR. Specifically, those skilled in the art can apply this modeling system to any process and/or detection technique in which stochastic noise is problematic. Thus, the optimal amount of any DNA degradation inhibitor can be determined based upon the number of DNA molecules that are sufficient to produce analyzable DNA.

Once the number of molecules for input to the PCR is determined, a sample comprising that number of molecules (or greater) is prepared for PCR according to standard methods. The number of molecules in a sample may be determined directly by, for example, enumerative methods such as those taught in U.S. Patent No. 5,670,325, incorporated by reference herein. Alternatively, the number of molecules in a complex sample may be determined by molar concentration, molecular weight, or by other means known in the art. The amount of DNA in a sample may be determined by mass spectrometry, optical density, or other means known in the art. The number of molecules in a sample derived from a biological specimen may be determined by numerous means in the art, including those disclosed in U.S. Patent Nos. 5,741,650 and 5,670,325, both of which are incorporated by reference herein.

Methods as described above are used to determine minimum or optimal amounts of DNA

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degradation inhibitors for use in any DNA isolation, detection, or amplification process in which stochastic processes occur. Using the above-described model for determining the minimum number of molecules that must be measured to reliably detect a low-frequency species, one can empirically determine how much of any given inhibitor should be used.

5 II. Example - Detection of DNA in Stool with EDTA as a DNA Degradation Inhibitor

A. Introduction

Methods of the invention are useful for analyzing DNA from stool to detect colorectal cancer. If colorectal cancer is diagnosed early, it may be treated effectively by surgical removal of the cancerous tissue. Colorectal cancers originate in the colorectal epithelium, and typically
10 are not extensively vascularized (and therefore not invasive) during the early stages of development. The transition to a highly vascularized, invasive and ultimately metastatic cancer which spreads throughout the body commonly takes ten years or longer. If the cancer is detected prior to invasion, surgical removal of the cancerous tissue is an effective cure. However, colorectal cancer is often detected only upon manifestation of clinical symptoms, such as pain
15 and black tarry stool. Generally, such symptoms are present only when the disease is well established, often after metastasis has occurred, and the prognosis for the patient is poor, even after surgical resection of the cancerous tissue. Early detection of colorectal cancer, therefore, is important because early detection may significantly reduce patient morbidity.

B. Experiments

20 The following experiments demonstrate that EDTA, an inhibitor of DNase, increases the yield of high-integrity DNA from a stool sample with a concomitant increase in the amount of amplifiable DNA. In these experiments, three aliquots of stool (5 g each) were homogenized in buffer (0.5 M Tris, 10 mM NaCl, EDTA). The buffer to stool ratio was 7:1; thus, 35 ml of buffer

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was used for each 5 g of stool. The buffer contained either 0 mM EDTA, 16 mM EDTA, or 96 mM EDTA. Each of the three aliquots was then diluted with additional buffer (not containing EDTA) to a final buffer to stool ratio of 20:1. Each aliquot was then centrifuged, and the supernatant, which carried the active DNA degrading fraction, was removed to a clean tube.

5 Then, a DNA mixture of 2 μ g *E. coli* DNA and 100 ng human genomic DNA was added to each tube. Each tube was incubated for 75 minutes at 37°C. Then, 42 μ l of Proteinase K and 250 μ l of 10% SDS (sodium dodecyl sulfate) were added to each tube followed by an overnight incubation at 37°C. After the overnight incubation, the DNA in each sample was prepared by standard techniques. *See, e.g.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY §§ 2.1-2.4 (Ausubel
10 *et al.*, 3d ed., 1995). Generally, a phenol extraction, a phenol/chloroform extraction, and a phenol extraction were performed prior to isolating the DNA. Then, the isolated DNA was placed into a standard Tris buffer.

Three experiments were conducted on the isolated DNA. The first experiment demonstrated that the DNA degrading activity present in homogenized stool supernatant is
15 inhibited by optimal amounts of EDTA, increasing the amount of high-integrity DNA. In this experiment, DNA was isolated from homogenized stool supernatant which was taken from aliquots of stool homogenized in buffer having 0 mM, 16 mM, or 96 mM EDTA. Total nucleic acid was run on a separation gel. Results are shown in Figure 2, where arrows identify the location of the smear (or lack thereof) containing the DNA of interest.

20 Lanes 4, 5, and 6 represent samples of DNA added to homogenized stool supernatant, obtained from stool homogenized in buffers containing 0 mM, 16 mM, or 96 mM EDTA, respectively, that was subsequently isolated. Note that each lane shows a high molecular weight band which represents endogenous DNA from the stool sample and a smear from the exogenous

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DNA. The intensity of the band and smear in the photograph (which correlates with the amount of DNA in the band, a greater intensity corresponding to a greater amount of DNA) increased as the concentration of EDTA in the original buffer increased. Lanes 7-9 and 10-12 are replicates of lanes 4-6. The increasing intensity of the bands and the smears as EDTA concentration increased indicated that DNA integrity was preserved as the concentration of EDTA in the buffer increased. Thus, the DNA degrading activity of the homogenized stool supernatant was inhibited by the EDTA in a roughly dose-dependent manner.

Lanes 1, 2 and 3 and lanes 13, 14 and 15 were control samples containing 2 μ g *E. coli* DNA and 100 ng exogenous human DNA in buffer made with 16 mM EDTA. As expected, each lane showed a smear representative of the added DNA.

In a second experiment, the isolated DNA as described above was captured and amplified. The results of this experiment demonstrated that EDTA not only inhibits the DNA degrading activity present in stool supernatant but also increases the amount of amplifiable DNA. In this experiment, after preparing the DNA as described above, a standard hybrid capture was performed using Kras-specific capture probes to capture Kras DNA. The Kras DNA then was PCR amplified. Figure 3 shows the effect of EDTA on the preservation of Kras DNA. The location of the band (or lack thereof) representing Kras DNA is identified with an arrow.

Lanes 4, 5, and 6 represent Kras DNA that was amplified from template DNA that was added to homogenized stool supernatant obtained from stool homogenized in buffer containing 0 mM, 16 mM, or 96 mM EDTA, respectively. Note that the Kras band in lane 4 was nearly absent, while the Kras band grew in intensity (representing an increase in the amount of Kras DNA actually present) in lanes 5 and 6 as the concentration of EDTA in the buffer increased. Lanes 7-9 are replicates of lanes 4-6 and show a similar increase in band intensity (an increase in

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the amount of DNA present) as the concentration of EDTA in the original buffer increased. Thus, more Kras DNA was amplified, resulting in a more robust signal at higher concentrations of EDTA.

Additionally, in the population, levels of DNA which can be amplified from stool vary across individuals. These individuals have been characterized in groups from A to F, with A being the highest level of DNA and F being an undetectable level of DNA. The high levels of DNA in group A are due to low DNA degradation activity in their stool ("high-integrity stool"). Adding EDTA to the buffer in which a stool aliquot from a Group A individual is homogenized would not be expected to produce a large effect because Group A stool has little DNA degrading activity. In fact, when Kras DNA was amplified from template DNA that was added to homogenized stool supernatant obtained from Group A stool homogenized in buffer containing 0 mM, 16 mM, or 96 mM EDTA, little difference in the amount of amplified Kras DNA was observed (lanes 10, 11, and 12, respectively). Only a slight increase in Kras band intensity can be seen between 0 mM EDTA and 16 mM or 96 mM EDTA, representing only a slight increase in the amount of Kras DNA at inhibitory concentrations of EDTA.

Lanes 1-3 as well as lanes 13-15 represent samples of amplified Kras DNA that was not exposed to homogenized stool supernatant. As expected, those control lanes show a band of equal intensity across lanes representing Kras DNA. Lanes 16 and 17 are negative controls and, as expected, show no band representing Kras, indicating that any observed Kras DNA is due to captured DNA and not to contamination. Lane 18 is a negative control, and, as expected, has no band representing the Kras gene, indicating that the PCR products are from the sample and not from contamination. Lanes 19-21 are positive controls where 50 pg, 100 pg, or 200 pg of human

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DNA is amplified in a background of *E. coli* DNA, indicating that human DNA can be amplified in an *E. coli* background in this model system. Finally, lane 22 is a molecular weight marker.

In a third experiment, the same protocol was used as in the second experiment except human genomic DNA was added into each sample after capture. Kras DNA was again amplified
5 by PCR. Thus, an excess of template DNA was available for PCR. This experiment demonstrated that the varying levels of PCR amplification in the second experiment were not due to EDTA interfering with or enhancing normal PCR but were due to varying levels of template DNA available to be amplified resulting from various levels of DNA degradation inhibition by EDTA. Figure 4 shows the results of this experiment. The location of the band (or
10 lack thereof) representing Kras is identified with an arrow. Lanes 1-6 and 8-13 correspond with lanes 1-12 in the second experiment (*i.e.*, lanes 1-3 were controls, lanes 4-6 and 8-10 were excess Kras DNA amplified in samples exposed to homogenized stool supernatant from stool homogenized in 0 mM, 16 mM, or 96 mM EDTA, and lanes 11-13 were excess Kras DNA amplified in samples exposed to homogenized stool supernatant from high-integrity stool
15 homogenized in 0 mM, 16 mM, or 96 mM EDTA). As expected, lanes 1-6 and 8-13 show a PCR product of roughly equal intensity because an excess of template DNA is available. The EDTA does not interfere with or enhance normal PCR. This result indicates that the varying levels of PCR amplification in the 0 mM, 16 mM, or 96 mM EDTA samples in the second experiment was due to varying levels of template DNA and not to inhibitor. Lane 14 shows a
20 sample of human genomic DNA. Lanes 15-18 are the same controls as lanes 17 and 19-21 in the second experiment.

From the experimental data described above, the amount of EDTA required to inhibit DNase was calculated. The concentration of EDTA in the various buffers used in the three

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experiments was normalized as grams of EDTA per gram of stool. Generally, the concentration of EDTA was multiplied by the molecular weight of EDTA and by the volume of buffer in which the stool was homogenized. The product was divided by the amount of stool that was homogenized. For example, the following equations were used to normalize EDTA

5 concentration.

For 16 mM EDTA:

$$(0.016 \text{ EDTA M/L} \times 372.2 \text{ g/M} \times 0.035\text{L}) \div 5\text{g} = 0.042 \text{ g EDTA per gram of stool}$$

For 96 mM EDTA:

$$(0.096 \text{ EDTA M/L} \times 372.2 \text{ g/M} \times 0.035\text{L}) \div 5\text{g} = 0.250 \text{ g EDTA per gram of stool}$$

10 Thus, for any amount of stool to be homogenized, at least about 0.042 g EDTA per gram of stool should be used in the homogenization buffer in order to maximize yield of DNA. The range of EDTA which may be used is from about 0.042 g EDTA per gram of stool to about 0.782 g EDTA per gram of stool. More preferably, about 0.250 g EDTA per gram of stool to about 0.521 g EDTA per gram of stool is used. Most preferably, about 0.391 g EDTA per gram of
15 stool is used.

These calculations indicate that at commonly used buffer volumes and stool amounts, the amount of EDTA present in the homogenized sample is a more important factor than the final concentration of EDTA in the homogenized sample. However, as one skilled in the art realizes, at some point, although the amount of EDTA will remain the same in a given volume, the
20 volume may become so large that the effect of EDTA on DNA integrity is diluted. When examining a stool sample within commonly used parameters, this dilution effect is not seen. However, in alternative embodiments, the concentration of EDTA is a relevant factor. In these embodiments, from about 16 mM EDTA to about 300 mM EDTA is useful. More preferably,

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from about 100 mM EDTA to about 200 mM EDTA is useful. Most preferably, about 150 mM EDTA is useful.

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What is claimed is:

- 1 1. A method for preserving the integrity of DNA in a stool sample, the method comprising:
2 exposing the stool sample to an amount of an inhibitor of DNA degradation sufficient to
3 produce a critical number of molecules of analyzable DNA.
- 1 2. The method of claim 1 wherein the inhibitor comprises an ion chelator.
- 1 3. The method of claim 2 wherein the ion chelator comprises a divalent ion chelator.
- 1 4. The method of claim 3 wherein the divalent ion chelator is selected from the group
2 consisting of EDTA and EGTA.
- 1 5. The method of claim 1 wherein the step of exposing the stool sample to an amount of an
2 inhibitor of DNA degradation comprises providing EDTA in a range from 0.042 g EDTA per
3 gram of stool to 0.782 g EDTA per gram of stool.
- 1 6. The method of claim 1 wherein the inhibitor of DNA degradation comprises an enzyme
2 inhibitor.
- 1 7. The method of claim 1 further comprising a step of extracting a target DNA.
- 1 8. The method of claim 7 wherein the step of extracting comprises a phenol-chloroform
2 extraction.
- 1 9. A method for preserving the integrity of DNA in a sample containing exfoliated cells, the
2 method comprising:
3 exposing the sample to a minimum amount of an inhibitor of DNA degradation sufficient
4 to produce a critical number of molecules of analyzable DNA.
- 1 10. The method of claim 9 wherein the sample is obtained from stool.
- 1 11. The method of claim 9 wherein the inhibitor comprises an ion chelator.
- 1 12. The method of claim 11 wherein the ion chelator comprises a divalent ion chelator.

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1 13. The method of claim 12 wherein the divalent ion chelator is selected from the group
2 consisting of EDTA and EGTA.

1 14. The method of claim 10 wherein the step of exposing the sample to a minimum amount
2 of an inhibitor of DNA degradation comprises providing EDTA in a range from 0.042 g EDTA
3 per gram of stool to 0.782 g EDTA per gram of stool.

1 15. The method of claim 9 further comprising extracting a target DNA.

1 16. The method of claim 9 wherein the step of extracting comprises a phenol-chloroform
2 extraction.

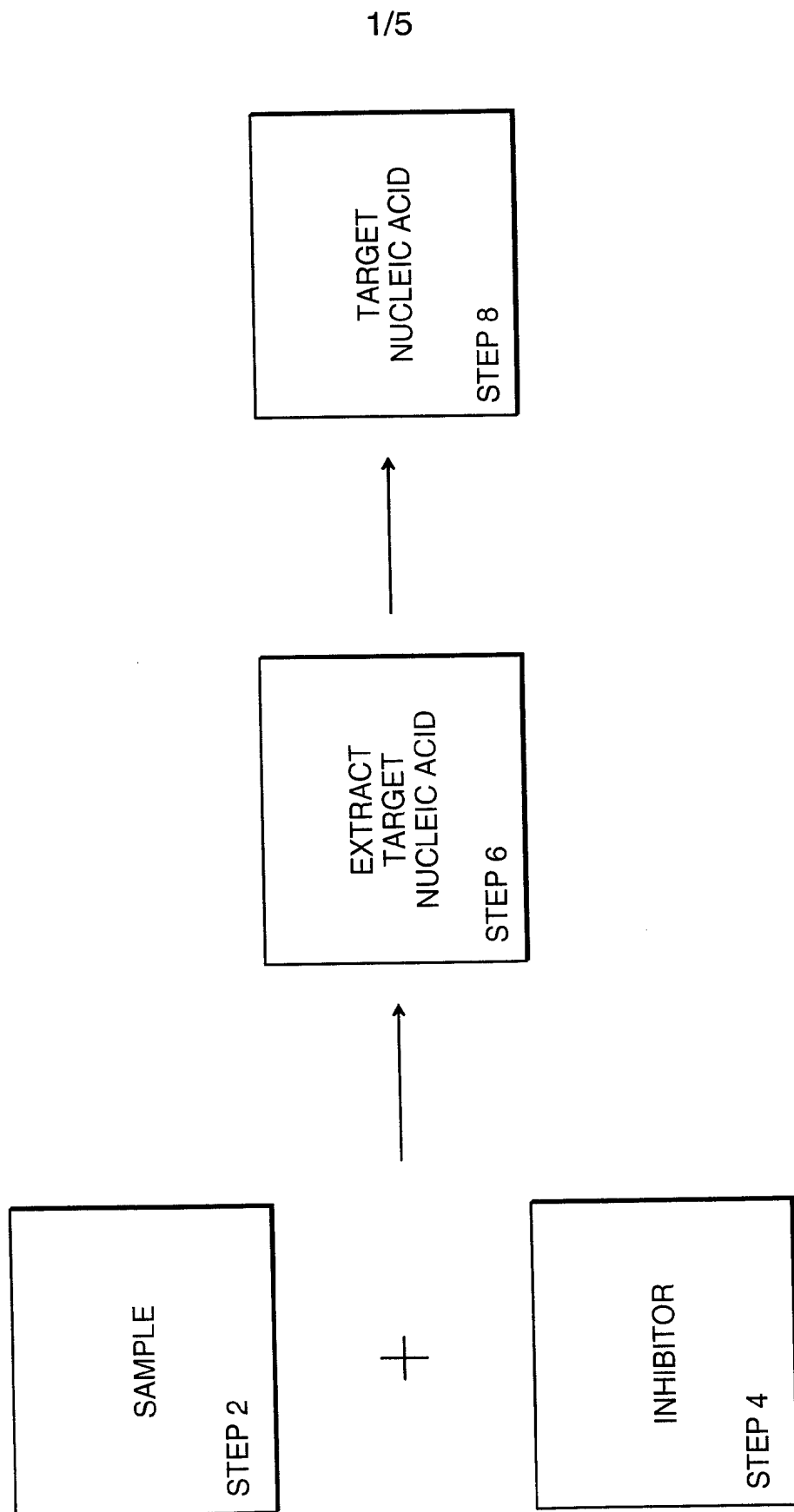


FIG. 1

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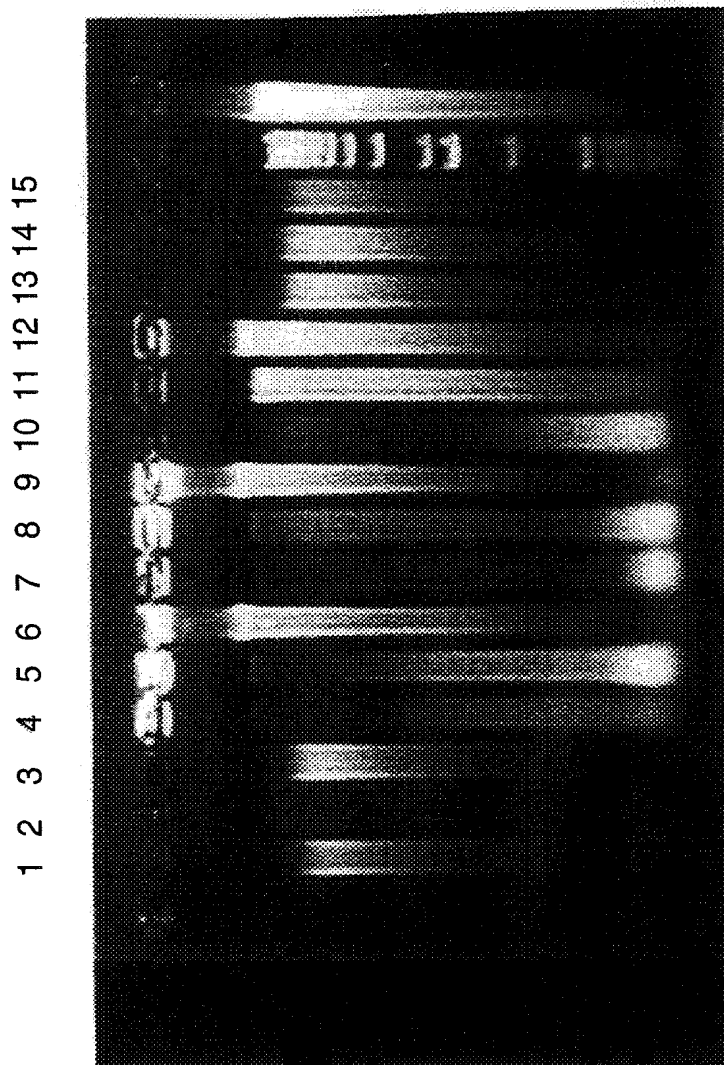


FIG. 2



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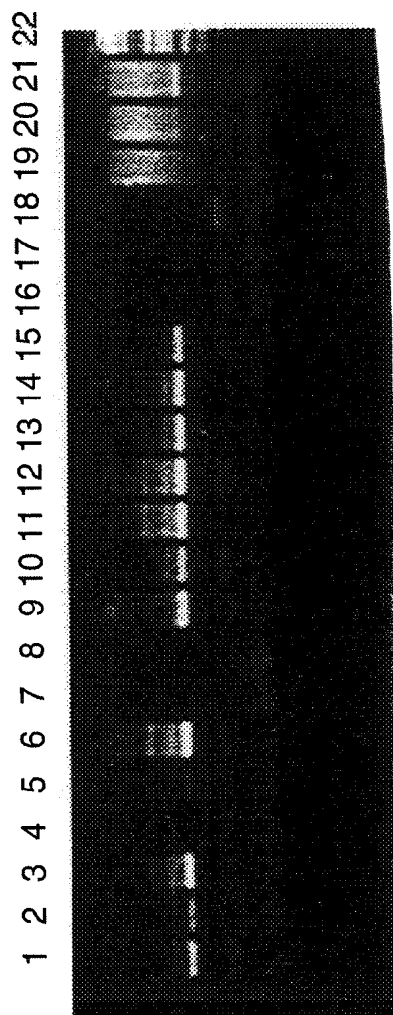
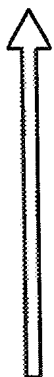


FIG. 3



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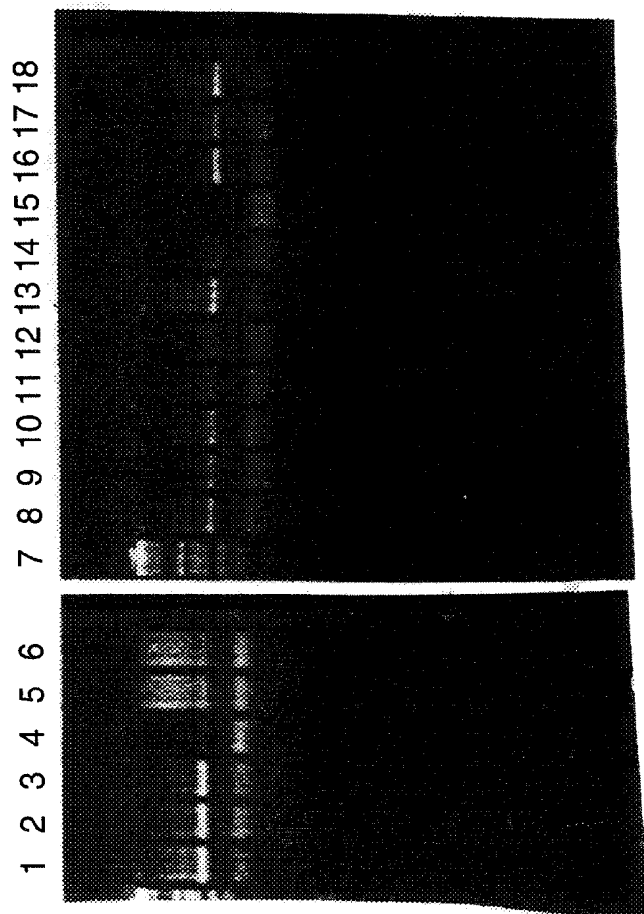


FIG. 4

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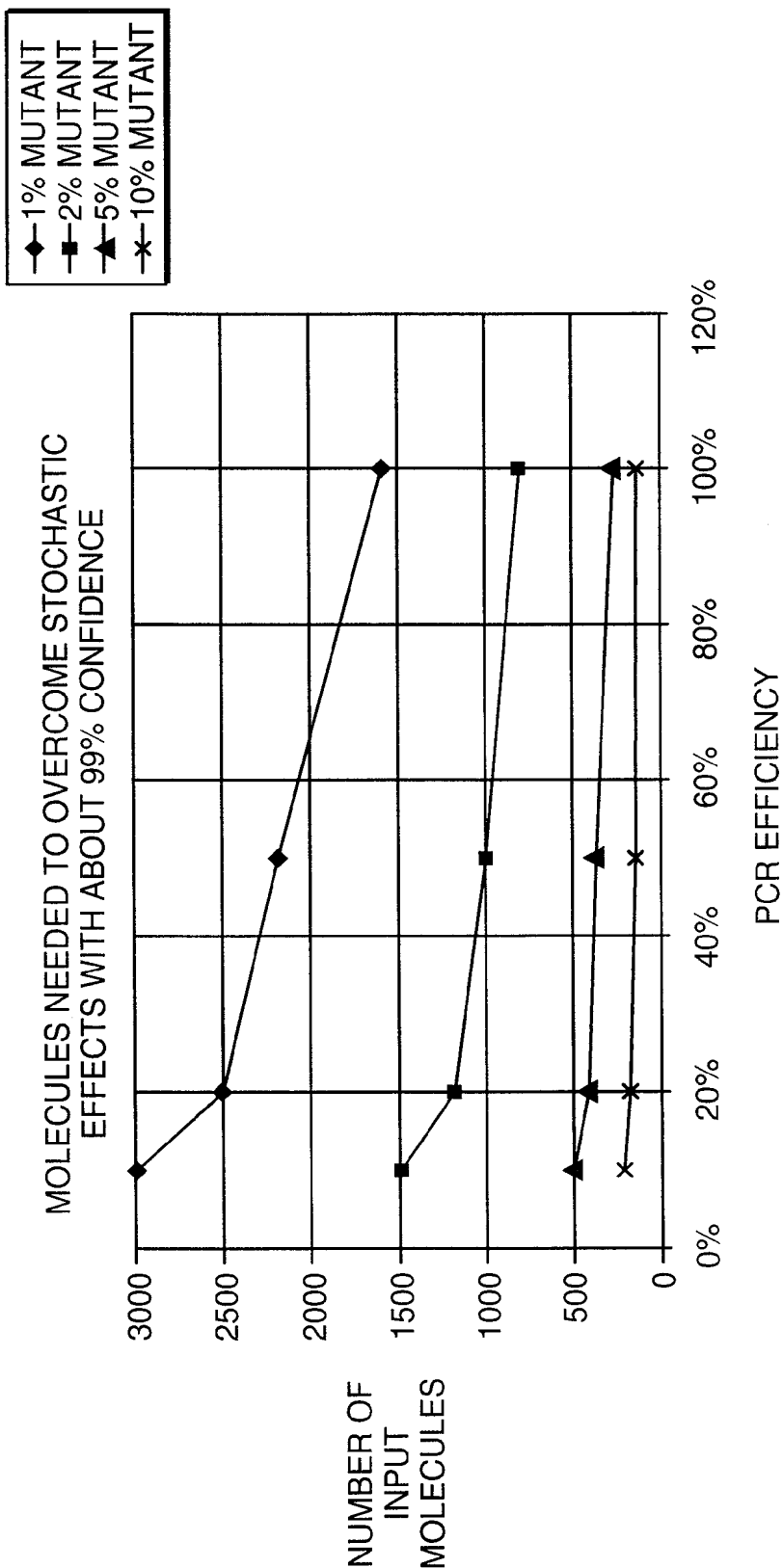


FIG. 5

INTERNATIONAL SEARCH REPORT

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PCT/US 00/01759

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12Q1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, MEDLINE, CHEM ABS Data, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 20235 A (UNIV JOHNS HOPKINS MED) 14 October 1993 (1993-10-14) the whole document ---	1-16
X	WO 98 58081 A (EXACT LAB INC) 23 December 1998 (1998-12-23) the whole document ---	1-16
X	DE 195 30 132 A (MAX PLANCK GESELLSCHAFT) 20 February 1997 (1997-02-20) the whole document ---	1-16
	-/--	
☒ Further documents are listed in the continuation of box C. ☒ Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 28 June 2000		Date of mailing of the international search report 04/07/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Hagenmaier, S

INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CALDAS C ET AL: "DETECTION OF K-RAS MUTATIONS IN THE STOOL OF PATIENTS WITH PANCREATIC ADENOCARCINOMA AND PANCREATIC DUCTAL HYPERPLASIA" CANCER RESEARCH,US,AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, vol. 54, 1 July 1994 (1994-07-01), pages 3568-3573, XP002050203 ISSN: 0008-5472 the whole document	1-16
X	SIDRANSKY ET AL.: "IDENTIFICATION OF RAS ONCOGENE MUTATIONS IN THE STOOL OF PATIENTS WITH CURABLE COLORECTAL TUMORS" SCIENCE, vol. 256, 1992, pages 102-105, XP000605488 the whole document	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/01759

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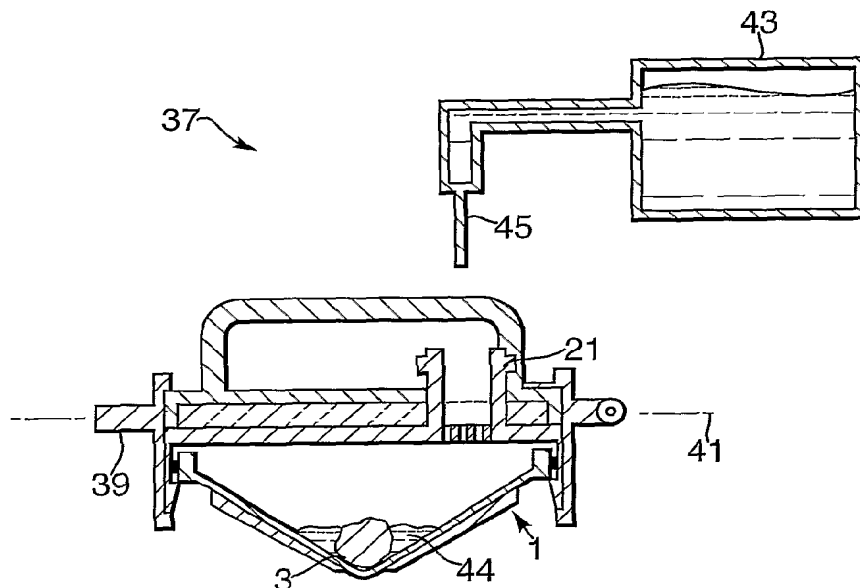
(72) Inventor; and

(75) Inventor/Applicant (for US only): **O'NEILL, IAN** [GB/GB]; 5 RED HILL CLOSE, GREAT SHELFORD, CAMBRIDGE Cambridgeshire CB2 5JP (GB).

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[Continued on next page]

(54) Title: METHOD AND APPARATUS FOR REMOVING CELLULAR MATERIAL FROM FAECAL STOOLS AND CONTAINER FOR COLLECTING FAECAL STOOLS



(57) Abstract: A method of removing cellular material from the surface of a faecal stool comprises the steps of: a) supporting the stool in a container having a channel along which the stool extends; b) cooling the stool to a temperature below its gel greezing point; c) introducing wash liquid into the container whilst maintaining the stool at a temperature below its gel freezing point; and d) moving the container to cause the liquid to run back and forth along the channel thereby washing the cellular material from the surface of the stool. Further disclosed are an apparatus (4) for removing cellular material from. the surface of a faecal stool and a container for collecting faecal stools (3b) .

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Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD AND APPARATUS FOR REMOVING CELLULAR MATERIAL FROM
FAECAL STOOLS AND CONTAINER FOR COLLECTING FAECAL STOOLS

Field of the Invention

5 The present invention relates to a method and apparatus for removing cellular material from faecal stools and a container for collecting faecal stools.

Background

10 Faecal stools are the residue of digestive products in the gastrointestinal tract, and contain not only bacteria and undigested food residues, but also residual products from the body's metabolism and structure such as exfoliated epithelial cells from the lining of the gastrointestinal tract.

15 Exfoliated 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
20 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. A. Loktionov *et al.*, in *Clinical Cancer*
25 *Research*, Vol. 4, 337-342, (1998), describe a non-invasive screening test for early detection of colorectal cancer which is based on a quantitative comparison of the DNA concentrations derived from exfoliated epithelial cells isolated from the surface of stools from healthy and
30 tumour-bearing persons. In this isolation procedure, cells on the surface of the stool were predominantly clustered and had varying degrees of intactness and damage. In the present specification by "cells" and "cellular material" we mean all such material irrespective of degree of clustering
35 or intactness.

In our earlier patent application, WO 97/09600, we describe a method of isolating cells (especially exfoliated epithelial cells) from the surface of faecal stools, comprising cooling a stool to a temperature (preferably of
5 -80°C to 15°C, preferably -10°C to 15°C, preferably -10°C to 10°C, more preferably 0°C to 5°C) below its gel freezing point and removing the cells from the cooled stool by agitating the stool in an aqueous solution (which may be pre-cooled to a temperature below the gel freezing point,
10 and is preferably ice-cold). The cooled condition creates a much more physically robust hard gel state that helps to prevent the stool from breaking up or rapidly eroding so that non-surface material is largely prevented from entering the aqueous solution.

15 Stool surface layers provide a particularly rich source of exfoliated cells suitable for isolation. Typically between one-sixth and one third of colonic epithelial cells are exfoliated per day (D. Sidransky et al. (1992), *Science* 256, 102-105 and W. C. MacDonald, J. S.
20 Trier, N. B. Everett, *Gastroenterology* (1964) 46, 405). It is believed that some of these exfoliated cells, together with mucus, may be envisaged as forming a "sheath" around a stool, containing a low proportion of bacterial cells and other faecal matter. It is further believed that the
25 viability of the cells in the surface layers (i.e. the layer of faecal material within 1.0 mm, and preferably 0.25 mm, of the stool surface) of the stool is facilitated by i) a tendency of the cells to agglomerate into plaques (which helps to prevent apoptosis), ii) the availability of oxygen
30 at the surface of the stool and iii) the unique ability of colonic epithelial cells to utilise as fuel the butyrate formed locally in high concentrations by microfloral fermentation (J. H. Cummings (1981) *Gut* 22, 763-799 and W. E. W. Roediger (1980) *Gut* 21, 793-798). Oxidation of
35 butyrate is believed to provide fuel for the cells in the

absence of blood-borne nourishment following exfoliation. It has been found that isolation of cells from stool surface layers provides significant advantages in yield, purity and ease of recovery over isolation of cells from homogenised stools.

Summary of the Invention

Although the method described in WO 97/09600 is effective for isolating cells from the surfaces of faecal stools, in order to make the method more suitable for high throughput screening of e.g. hundreds or thousands of samples per day, problems remain of washing the stools in a standard and reproducible way that maximises the amount of surface cells extracted from the stools but minimises the risk of breaking up the stools.

Consequently, in a first aspect, the present invention provides a method of removing cellular material from the surface of a faecal stool comprising the steps of:

- a) supporting the stool in a container having a channel along which the stool extends;
- b) cooling the stool to a temperature below its gel freezing point;
- c) introducing wash liquid into the container whilst maintaining the stool at a temperature below its gel freezing point; and
- d) moving the container to cause the liquid to run back and forth along the channel thereby washing the cellular material from the surface of the stool. A minority of stools are very soft when excreted, such that on cooling they do not reach the gel freezing point, but do become sufficiently viscous to be washed with minimal entry into the wash liquid of sub-surface material. Thus, for that minority of stools, by "gel freezing point" we mean the temperature at which the stools become sufficiently viscous to be washed with negligible entry of sub-surface material into the wash liquid.

The method promotes the reproducible and gentle washing of the stool, which facilitates the quantitative comparison of cellular material between individuals.

Human faecal stools are typically elongate and
5 approximately cylindrical in shape. So the channel significantly reduces the likelihood that sections of the stool will break off when the stool is being washed because the channel walls substantially prevent side-to-side
10 rolling motion of the stool and, for a wide range of rocking angles and speeds, friction largely prevents the stool from sliding along the channel. Preferably the container is moved to cause a wash frequency of from 5 to 40 back and forth cycles per minute, and more preferably from 15 to 25 cycles per minute. The stool may be pre-
15 soaked (for e.g. 3 minutes) in the wash liquid prior to step d), in which case preferably the container is moved for a total time of from 10 seconds to 1 minute. Without a pre-soak, preferably the container is moved for a total time of from 30 seconds to 5 minutes. With or without pre-
20 soaking it is advantageous to limit the exposure of the stool to the wash liquid so as to avoid localised depletion of wash reagents or over-enrichment of released cellular material. The channel may have a V or U-shaped transverse section.

25 An advantage of the container motion, is that the wash liquid is channelled back and forth along the length of the stool so that, compared with e.g. circular agitation by an orbital shaker of the wash liquid relative to the stool (which we have found in any event tends to break up the
30 stool), less liquid is required to wash the cells from the surface of the stool. Clearly the less liquid that is used to wash the stool, the higher the resulting concentration of cells in the liquid, which can in turn improve the accuracy of screening tests which may be subsequently
35 performed on the liquid. Also smaller volumes of liquid

reduce cost and disposal problems. Preferably from 30 to 150 ml of liquid is used in steps c) and d). This is generally sufficient to wash a typical 100 g stool while minimising losses due to residual wetting of the container and stool. Most preferably a fixed ratio of liquid to stool is used so that all stools experience a similar degree of wetting. This ratio may be in the range 0.5 to 1.0 ml of liquid per gram of stool.

In one embodiment, step d) comprises rocking the container about a horizontal axis perpendicular to the channel so that the liquid runs back and forth under gravity. In another embodiment, step d) comprises displacing the container back and forth in the direction in which the channel extends.

Preferably the container is of fixed shape, e.g. by being formed of rigid material.

In a second aspect, the present invention provides an apparatus for removing cellular material from the surface of a faecal stool, comprising:

mounting means for mounting a container having a channel along which, in use, the stool extends; and

motion imparting means for moving the container so that, when wash liquid is introduced into the container, the liquid runs back and forth along the channel thereby washing cellular material from the surface of the stool.

In one embodiment, the motion imparting means rocks the container about a horizontal axis perpendicular to the channel so that the liquid runs back and forth under gravity. In another embodiment, the motion imparting means displaces the container back and forth in the direction in which the channel extends.

The apparatus may further comprise control means to control e.g. the rocking angle, rocking or displacement frequency and/or total time of movement of the container, preferred ranges for some of these parameters being

provided above in respect of the first aspect of the invention. The control means may further control a sequence of stationary and mobile periods for the container, so that e.g. stationary exposure of the stool to the wash liquid allows the liquid to penetrate the stool and loosen its surface layers prior to active washing when the container is moved.

With such an apparatus, stool washing can be performed in a repeatable, standardised manner, and with a relatively high stool sample throughput. These are significant advantages if the wash liquid is to provide the material for a screening test based on e.g. a quantitative comparison of amounts of epithelial cell components.

The apparatus may further comprise cooling means for cooling the stool to a temperature (preferably of -80°C to 15°C , preferably -10°C to 15°C , preferably -10°C to 10°C , more preferably 0°C to 5°C) below its gel freezing point.

In one embodiment the apparatus further comprises a filter means such as a sieve, so that, when wash liquid is removed from the container, the cells are retained in the liquid but food residues from the stool are filtered from the liquid.

The apparatus may further comprise a metering device for introducing a metered dose (e.g. 50 to 150 ml, or an amount which is related to the stool weight) of wash liquid into the container, and/or a wash liquid pre-cooler for cooling the wash liquid to a temperature below the gel point of the stool before the wash liquid is introduced into the container. This temperature is preferably about 0°C .

The apparatus may further comprise at least one and preferably a plurality of detachable sample receptacles into which wash liquid is transferable from the container, so that e.g. when stool washing is completed the wash liquid may be poured from the container into the

receptacles. These can then be detached from the apparatus and transferred to an automated liquid-handling system for high throughput processing of the liquid samples contained therein.

5 A third aspect of the invention provides a fixed shape container for collecting faecal stools which has an upper receiving mouth and a lower stool collection channel, the internal walls of the container narrowing from the mouth to the channel so that when a stool is deposited through the
10 mouth the stool is encouraged to lie lengthways along the channel. The mouth can be of any suitable shape, e.g. rectangular or circular. The length of the channel is preferably in the range from 10 to 25cm, which corresponds to the size of a typical stool. The container may be used
15 in the first or second aspect of the invention.

 As faecal stool is generally regarded as disgusting material that people are reluctant to handle, even indirectly, the fixed shape container has several advantages as compared with, e.g. a flexible plastic
20 specimen bag for collecting stool. For example, the mouth of the container cannot collapse as a stool is being deposited in the container, thereby making it easier for a user to excrete into the container. Also the container can be handled without, apart from the weight of the stool, any
25 sensation that a stool is contained therein.

 Furthermore the funnelling effect of the narrowing container walls guides a formed stool (which on defecation can be quite malleable) to a position in which it extends along the channel. This reduces or eliminates the need for
30 the user to directly or indirectly manipulate the stool into that position prior to stool washing. Soft stools, and even diarrhoea, are also encouraged to align along the channel by the narrowing of the container walls.

 A further advantage of the container is that it can
35 serve both as the receptacle into which the stool is

excreted and as the container in which the stool is washed. So the stool does not need to be transferred to a different washing container, which avoids damage to the stool surface caused by such a transfer. Such damage would interfere
5 with the accuracy of e.g. screening tests which may be performed on the stool.

If 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
10 reasonably securely in the channel so that transportation damage to the stool surface is reduced or avoided.

To avoid user embarrassment during transportation at least a majority of the container may be opaque (i.e. a stool inside the container is not visible through the
15 opaque portions of the container, even if the material of the container transmits light to some degree), or an opaque transport casing may be provided for transporting the container. However, desirably at least a portion of the container permits viewing to the inside of the container so
20 that e.g. after delivery of the container for stool processing a health professional can check the stool for gross abnormalities such as blood before the stool is processed and without opening the container.

The container may further have installation means
25 (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
30 usual manner, which promotes user acceptability.

Typically, for hygiene reasons, at least the stool-contacting parts of the container are disposable. However, in one embodiment the internal walls of the container serve as a former for a bag which is reversibly fixable inside
35 the container, whereby the bag assumes the shape of the

mouth and channel. In this embodiment the bag is typically disposed after use, but the container can be reused.

Preferably the container is made of plastics material such as polypropylene. Desirably the container has smooth
5 internal surfaces which reduce damage to the surface of the stool as the stool passes from the mouth to the channel.

In one embodiment the container has cooling means, such as an outer jacket or bath, for cooling a stool deposited in the container. This means that the stool can
10 be cooled immediately it is produced, which helps to preserve the surface layer cells and makes the stool robust for physical transport. The cooling means may be of material that can be pre-cooled in a domestic refrigerator or freezer. This is particularly convenient when the stool
15 is produced at home, allowing the donor to defecate at a convenient time and in a minimally stressful setting and thereby promoting user acceptability. The cooling means may enable the stool to be cooled to a temperature below its gel freezing point.

20 The cooling means may be detachable from the container so that the cooling means can be pre-cooled before fitting to the container. Also, if the container is disposable, the cooling means can still be reused.

Anaerobic bacteria in stools produce damaging radicals
25 when exposed to atmospheric oxygen (C. F. Babbs (1990) *Free Radical Biology and Medicine*, 8, 191-200). Such oxygen also oxidises the mucus on the surface of stools and encourages the growth of aerobic bacteria. So preferably the container is hermetically sealable to reduce the
30 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. A sealed container rapidly becomes anaerobic due to the high concentration of anaerobic bacteria in the stool,
35 and this apparently largely prevents the growth of aerobic

bacteria on the stool surface, helping to preserve the stool. We have found that sealed containers allow stools to be stored at 0°C for a number of weeks apparently without significant degradation. To promote user
5 acceptability, the seal should also eliminate any odour from the stool escaping from the container, particularly if the stool is produced at home and then transported before washing.

The container may also have a liquid port which allows
10 wash liquid to be removed from the container while maintaining the stool in the container. For example the diameter of the port may be sized to prevent a typical-sized stool from exiting via the port, or a foraminous screen over the port may serve the same function. The port
15 may include closure means such as a valve or a cap which allows the liquid to be poured or sucked out of the container but at other times maintains an airtight seal. The same or another port may be used to introduce wash liquid into the container.

20 A further aspect of the invention provides a kit of parts comprising the container of the previous aspect with the installation means and/or the cooling means and/or the transport casing.

Another aspect of the invention provides a method of
25 screening for unhealthy condition(s) of the epithelium (such as colorectal cancer, polyps and ulcerative colitis) in a plurality of subjects (which are typically human subjects, but may be other animal species such as dogs and cats), the method comprising the steps of:

30 a) providing to the subjects respective containers (e.g. according to the third aspect of the invention) for defecation of a stool sample thereinto at the convenience of the subject (which is typically when the subject is "at home" or in a domestic setting);

b) collecting the filled containers from the subjects at a central location (which may be a doctor's surgery or a hospital out-patients clinic);

5 c) removing a specimen of cellular material from the surface of each stool sample (e.g. according to the first aspect of the invention); and

d) testing each specimen for unhealthy condition(s) of the epithelium (e.g. as described by A. Loktionov *et al.*, referenced above).

10 Brief Description of the Drawings

The present invention is now described in respect of a specific embodiment and with reference to the accompanying drawings in which:

15 Figs. 1a and b show schematically and respectively a cross section through and a plan view of a receptacle for collecting a faecal stool, and Fig. 1c shows a plan view of an alternative form of the receptacle;

Fig. 2 shows the same cross section as Fig. 1a except that a lid is fixed to the receptacle;

20 Fig. 3a shows a schematic cross section of the receptacle of Fig. 1a fixed inside a transport unit, and Fig. 3b shows the receptacle of Fig. 1c fixed in another version of the transport unit;

25 Fig. 4 shows schematically a stool washing apparatus with the receptacle of Fig. 1a positioned in a sample rack; and

Fig. 5 shows the apparatus of Fig. 4 with the sample rack pivoted to pour wash liquid out of the receptacle.

Detailed Description

30 Figs. 1a and b show respectively a cross section through and a plan view of a transparent plastic receptacle 1 having a V-shaped cross-section for collecting a faecal stool 3 produced by a user. The receptacle has a mouth 5 and a straight stool collection channel 7 (which extends
35 perpendicularly to the plane of the paper in Fig. 1a). The

receptacle is removably installed inside a toilet bowl 9 by a support web 11 which is attached over the rim of the toilet and has a central hole 12 to receive the receptacle so that channel 7 is horizontal and mouth 5 is directly
5 above channel 7. The smoothly sloping walls 14 of the receptacle guide the stool to the bottom of channel 7 and encourage the deposited stool to align with the longitudinal direction of the channel.

Fig. 1c shows an alternative form of the receptacle
10 which has a U-shaped channel cross-section, and which is capable of receiving larger specimens.

Once the stool is collected in the receptacle, a lid 13 (see Fig. 2) is used to close mouth 5 and is releasably fixed to the receptacle by a snap-fit mechanism 16. A
15 sealing ring 15 seals the receptacle from the external atmosphere.

Lid 13 includes a carrying handle 17, thermally insulated upper wall 19 and wash liquid entry/exit port 21. The port is sealed by closure cap 23 and has a foraminous
20 screen 25 at its inner end which prevents the stool from entering the port when (as described below) wash liquid is decanted from the receptacle, and a coarse sieve 25a to retain particles of e.g. greater than 2 mm in diameter.

The receptacle is lifted by handle 17 from web 11 and
25 fixed inside a transport unit 27 by a snap-fit mechanism 29 (see Fig. 3a). The transport unit has thermally insulated walls 31 and contains a cooling block 33 of pre-cooled ice or chemical cooling medium contained under a shaped divider 35. The cooling block is brought into close proximity with
30 the outer wall of receptacle 1. Ring 15a maintains the position of channel relative to the cooling block. The cooling block cools stool 3 to a temperature preferably in the range from 0 to 5°C, which is below the gel freezing point of the stool.

Fig. 3b shows the receptacle of Fig. 1c fixed in another version of the transport unit. In this version divider 35 forms an air space around the receptacle. The air space and divider 35 prevent the cooling block cooling the stool so rapidly as may damage the outer surface layers of the stool.

The stool is then transported in the transport unit to e.g. a clinic where stool washing is performed. The walls of the transport unit and lid are opaque so that the stools are not visible inside the unit, and lid 13 prevents odours from escaping the unit. Channel 7 also reduces movement of the stool during transportation which prevents significant damage to the surface of the stool.

At the clinic, the receptacle 1 and lid 13 are removed from the transport unit. As the receptacle is transparent visual inspection of the stool is possible without removing the lid. At this stage the stool sample may be rejected if, for example, it is too small or runny. also any gross evidence of disease, such as blood, may be noted. However, if the stool is acceptable, the receptacle and lid are positioned into the sample rack 39 (i.e. receiving means) of a stool-washing apparatus 37 shown in Fig. 4. Collection channel 7 is aligned perpendicular to axis 41 about which rack 39 is pivotable and which at this stage is horizontal.

Cooling means (not shown), such as a cold air jet, refrigerator unit or coolant bath, keeps or brings the stool to a temperature in the range 0 to 5°C so that it is below its gel freezing point.

Apparatus 37 has a reservoir 43 of chilled wash liquid, such as a buffered aqueous solution containing N-acetylcysteine, bovine serum albumin and sodium butyrate which has been found (see WO 97/09600) to improve recovery of exfoliated cells. When the stool is sufficiently cool, closure cap 23 is removed and a metered dose of wash liquid

44 is injected into the receptacle via an injector 45 and port 21. The liquid may alternatively be injected through an air-tight septum at port 21. Afterwards injector 45 is swung away from the sample rack.

5 Rocking means (not shown), which is e.g. an electric motor and associated drive, then rocks the sample rack and receptacle backwards and forwards about axis 41. A control unit (also not shown) which controls the rocking means defines a wash programme consisting of rocking angle,
10 rocking frequency and total exposure and rocking time experienced by the stool. As receptacle 1 moves, gravity causes wash liquid 44 in the receptacle to run back and forth along channel 7 so that intimate contact with the stool is achieved and surface cells from the stool are
15 washed into the liquid. To a significant extent friction prevents the stool from moving during the rocking procedure.

 When the wash programme ends, the sample rack is pivoted about pivot 47 to bring axis 41 into a vertical
20 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
25 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
30 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
35 receptacle 1 is removed and safely disposed of.

In this way, the apparatus converts a typical total 100-500 g mass of stool and wash liquid into aliquots of cell-suspension of typically only 50-1000 mg (i.e. a thousand-fold reduction in scale). The aliquots can be made representative by preventing the cells from settling out during the decanting process the aliquots. One method of preventing settling out is to provide mixing channels (not shown) through which the liquid flows on its way to the sample receptacles. Alternatively a stirrer (also not shown) can be provided to stir the wash liquid. Fine sieves (e.g. of water wettable material such as nylon, and/or having a mesh size in the range 50-400 μm , preferably 100-250 μm , more preferably 125-200 μm) may be provided over the entrances to the receptacles to further filter the liquid. The excess liquid flows to waste.

The samples may be used for e.g. DNA assays as described in WO 97/09600 and A. Loktionov *et al.*, *Clinical Cancer Research*, Vol. 4, 337-342, (1998).

The entire procedure, from when the receptacle is positioned in the sample rack to when the wash liquid exits the receptacle, can be automated, with standard wash programme settings being used. Also no manual contact with or manipulation of the stool required, which promotes good hygiene. So the present invention allows a large number of stool samples to be processed in a safe and reproducible manner.

In an experimental study, stools were collected into re-sealable bags and stored at 0°C from no more than a few minutes after excretion. The bags were transparent, which allowed visual inspection of the stools during testing. For stool washing, a wash solution was poured into each bag and the bag was located inside a rigid rocking channel of a stool washer apparatus, the channel being 40-45mm wide and perforated so as to permit intimate cooling by surrounding

ice-water held in an insulated container equipped with a submersible pump. Rocking caused a gravity-imposed back and forth circulation of the wash solution along the channel and also promoted cooling of the stool by the surrounding ice-
 5 water. A frequency of 3 seconds per rocking cycle was chosen.

The 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
 10 clean-up was performed robotically on a TECANTM 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 TECANTM Spectrafluor Plus using PicoGreen
 15 (Molecular ProbesTM) 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.

20

Effect of pre-soaking on SDNAI

The following SDNAI data from the stool washer (using a +/-45deg rocking angle) demonstrate the advantage of a period of pre-soaking. Presoaking appears to promote a
 25 more or less equilibrated suspension state of cellular material in the wash solution, which thereby helps to prevent the chance dynamic release of cellular material.

Rocking time	SDNAI (ng/ml) with no pre-soak			SDNAI (ng/ml) with 3 min pre-soak		
	stool 1	stool 2	stool 3	stool 4	stool 5	stool 6
30-40 sec	34	12	29	115	206	217
1 min	118	43	21	101	221	294
3 min	100	80	75	125	216	338

Effect of Rocking Angle

If too small a rocking angle was used or the channel became obstructed by the stool, the wash liquid tended to form non-flowing pools that failed to contact the stool surface so that unacceptable variations in washing condition were experienced. However, under favourable conditions, reproducible and relatively constant results could be obtained over a range of rocking angles. For example in three different experiments the same stool was given a 3 minute pre-soak followed by a 1 minute of wash at different rocking angles in a visibly free flow of liquid in a channel 40-45 mm wide and using a wash liquid to stool ratio of 0.7 ml per gram. The rocking angles for the three experiments were +/-30, 45 and 60° from the horizontal and the respective SDNAI measurements were 263, 254 and 260 ng/ml.

Effects of Damage to Stool Surface Layer and of Stool Age

Sometimes stored stools were found to be accidentally squashed, or stored in improperly sealed containers as evidenced by smalls amount of white bacterial over-growth on the stools. The following data (using different stools all washed for 1 minute after a 3 min pre-soak at a wash solution/stool weight ratio of 0.7) demonstrate the importance of protecting the stool surface layer during storage. However, they also demonstrate that under favourable conditions storage durations of up to several weeks have no particular effect on measured SDNAI values. The squashed stools had SDNAI values significantly above the 700 ng/ml value which is considered to be the upper limit for healthy persons.

Stool age (days) donor A	1	4	7	11 ^s	15	19	81 ^s
SDNAI	203	192	90	1,400	218	585	987

Stool age (days) donor B	14	29 ^p	29 ^p	31	42 ^p	73	101
SDNAI	255	23	50	177	78	261	515

^s indicates the stool was squashed

^p indicates the stool had bacterial over-growth

Effect of Wash Solution Volume

5 Each of a series of undamaged stools from one donor was washed (with a 3 min pre-soak and a 3 min wash at +/- 45° rocking angles) with both a fixed 50 ml volume of wash solution and a variable volume of wash solution based on a 0.5 ml/gram wash/stool ratio. For successive stools of the

10 series, washing first was alternated between the fixed and variable volume. The variable volume based on the 0.5 ratio resulted on average in the use of a 64% greater volume of wash solution which visibly appeared to wet the whole stool better and produced a 21% greater DNA

15 concentration. The second washes gave a 54% higher on average DNA yield than the first washes (largely irrespective of whether the fixed or variable wash solution volume was used first) which was presumably due to the pre-softening effect of the first washes. In general therefore

20 the fixed ratio wash took better account of the stool weight without significantly influencing the DNA result. As shown above, stool age had an insignificant effect on the results.

stool age (days)	1	5	9	20	60	70
stool weight (g)	89	141	103	232	274	146
SDNAI from 50 ml wash	1 st 55	2 nd 167	1 st 234	2 nd 300	2 nd 256	1 st 49
SDNAI from 0.5 ratio wash	2 nd 139	1 st 92	2 nd 220	1 st 262	1 st 207	2 nd 65

Claims:

1. A method of removing cellular material from the surface of a faecal stool comprising the steps of:

- 5 a) supporting the stool in a container having a channel along which the stool extends;
b) cooling the stool to a temperature below its gel freezing point;

10 c) introducing wash liquid into the container whilst maintaining the stool at a temperature below its gel freezing point; and

d) moving the container to cause the liquid to run back and forth along the channel thereby washing the cellular material from the surface of the stool.

15

2. A method of removing cellular material according to claim 1, wherein step d) comprises rocking the container about a horizontal axis perpendicular to the channel so that the liquid runs back and forth under gravity.

20

3. A method of removing cellular material according to claim 1, wherein step d) comprises displacing the container back and forth in the direction in which the channel extends.

25

4. A method of removing cellular material according to any one of claims 1 to 3, wherein the container has a fixed shape.

30 5. An apparatus for removing cellular material from the surface of a faecal stool, comprising:

mounting means for mounting a container having a channel along which, in use, the stool extends; and

35 motion imparting means for moving the container so that, when wash liquid is introduced into the container,

the liquid runs back and forth along the channel thereby washing cellular material from the surface of the stool.

5 6. An apparatus for removing cellular material according to claim 5, wherein the motion imparting means rocks the container about a horizontal axis perpendicular to the channel so that the liquid runs back and forth under gravity.

10 7. An apparatus for removing cellular material according to claim 5, wherein the motion imparting means displaces the container back and forth in the direction in which the channel extends.

15 8. An apparatus for removing cellular material according to any one of claims 5 to 7, further comprising cooling means for cooling the stool to a temperature below its gel freezing point.

20 9. An apparatus for removing cellular material according to any one of claims 5 to 8, further comprising filter means which filters food residues from the stool but retains the cellular material in the wash liquid when the liquid is removed from the container.

25 10. An apparatus for removing cellular material according to any one of claims 5 to 9, in which the container is mounted at the mounting means.

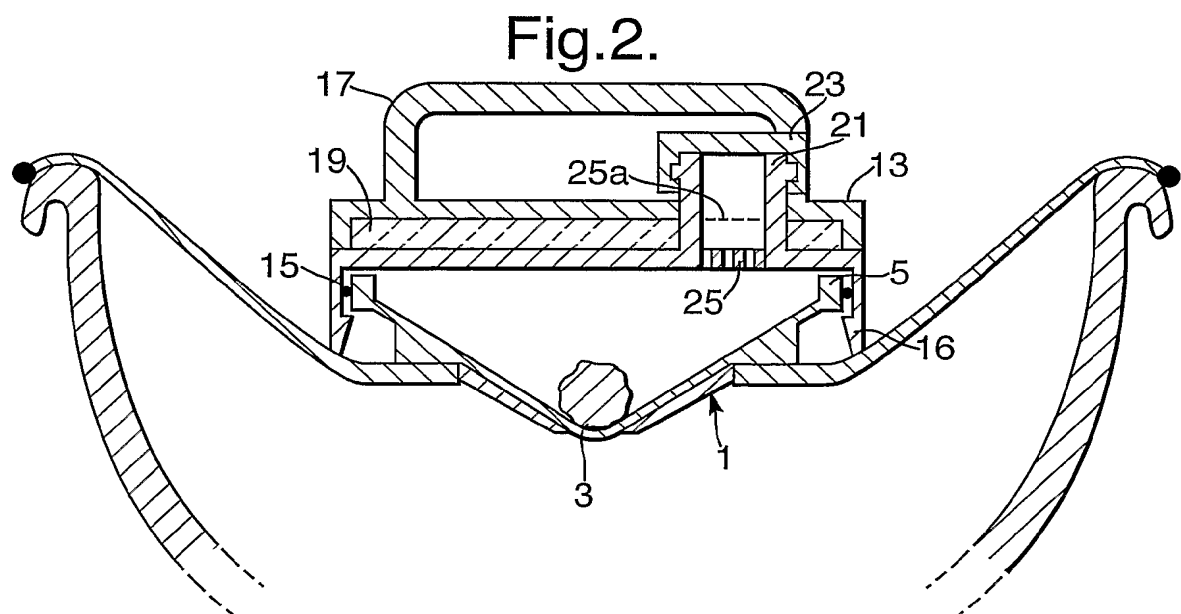
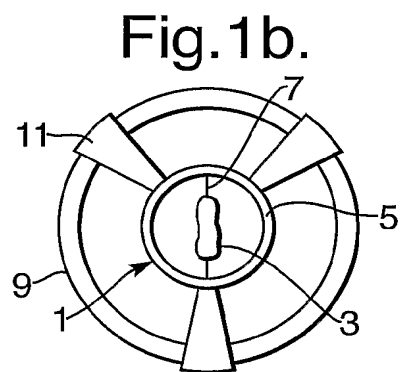
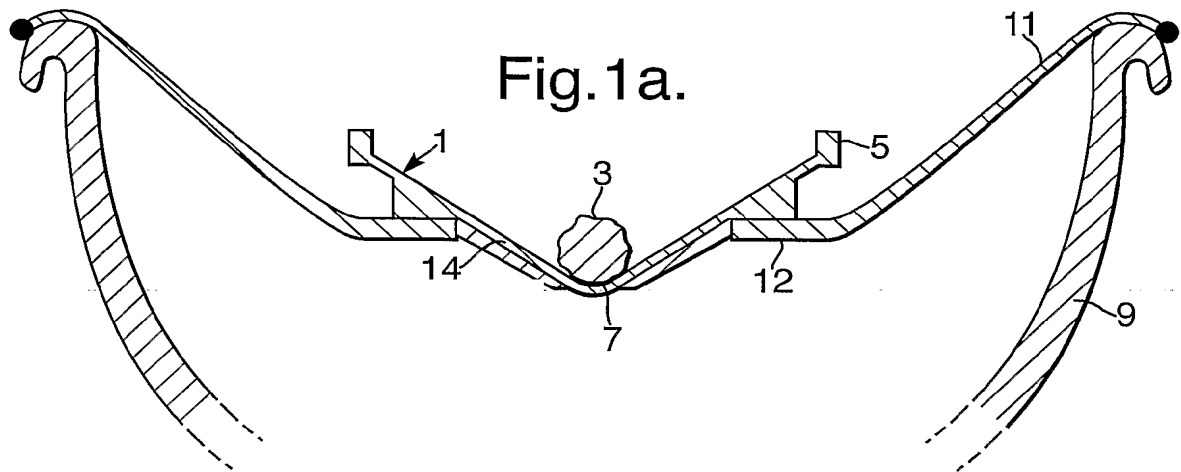
30 11. A container for collecting faecal stools which has a fixed shape and has an upper receiving mouth and a lower stool collection channel, the internal walls of the container narrowing from the mouth to the channel so that when a stool is deposited through the mouth the stool is
35 encouraged to lie lengthways along the channel.

12. A container according to claim 12, further having
installation means for removably installing the container
on a toilet with the receiving mouth opening upwardly and
5 the collection channel extending horizontally beneath the
mouth.

13. A container according to claim 11 or 12, further
having cooling means for cooling a stool deposited in the
10 container.

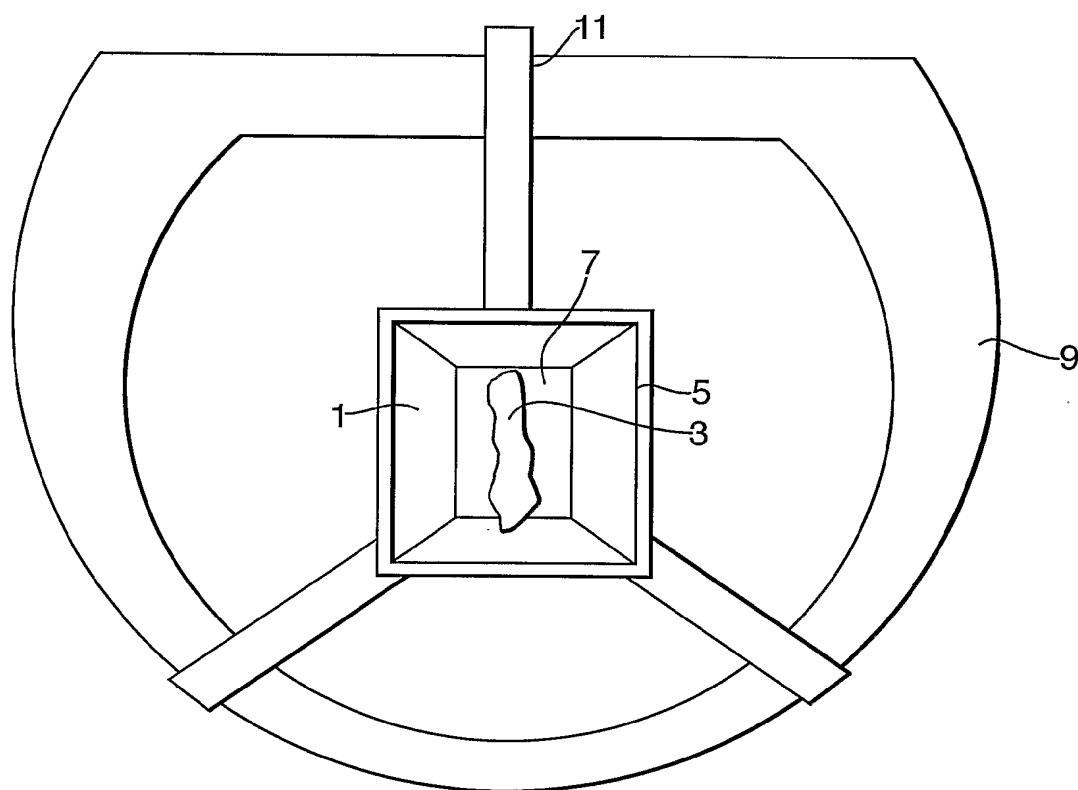
14. A container according to any one of claims 11 to 13,
which is hermetically sealable to reduce the exposure to
atmospheric oxygen of a stool deposited therein.
15

1/4



2/4

Fig.1c.



3/4

Fig.3a.

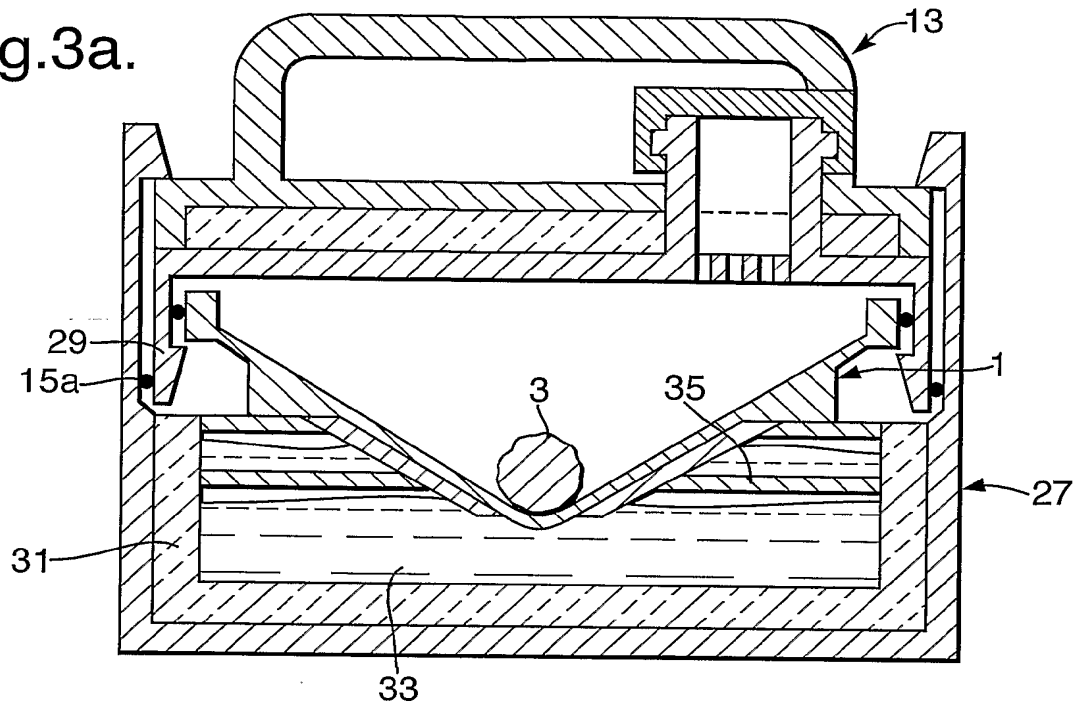
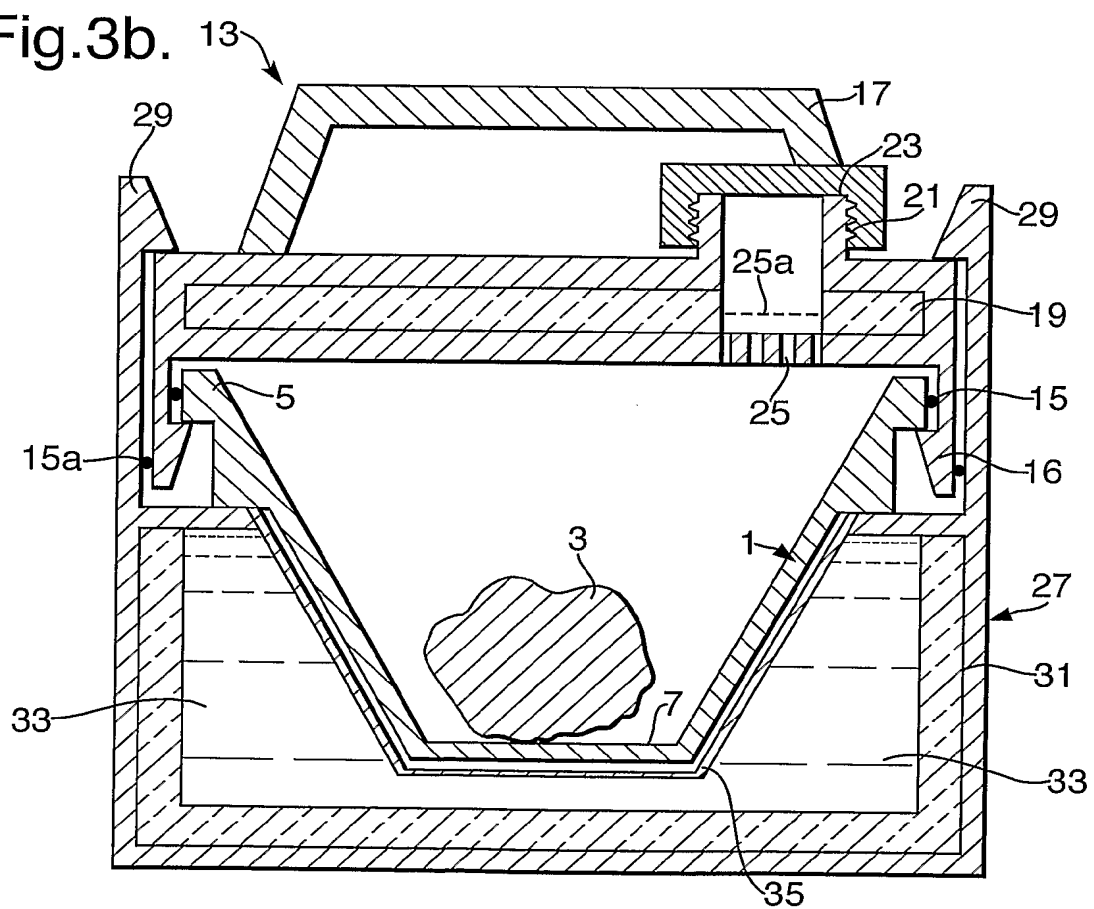
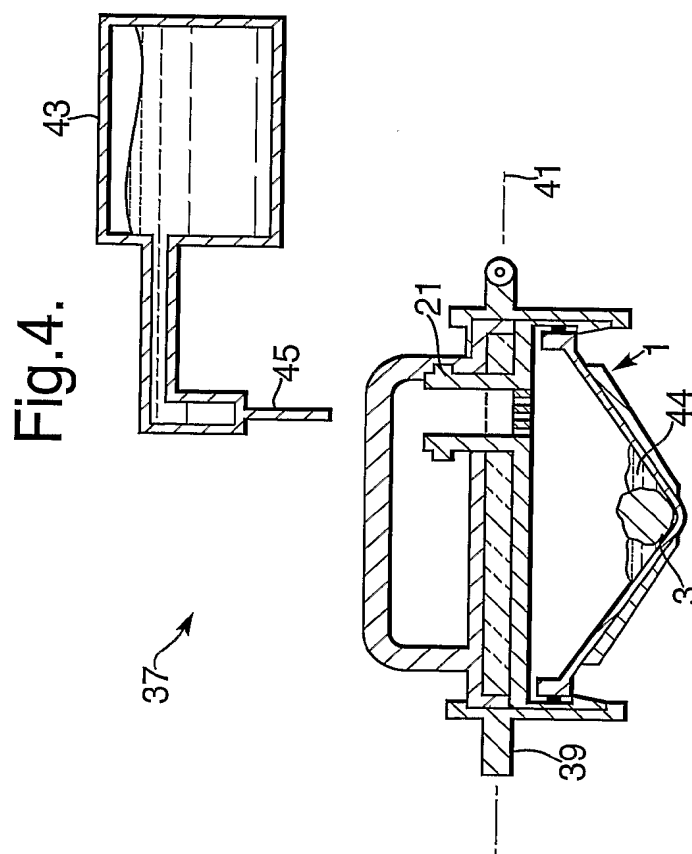
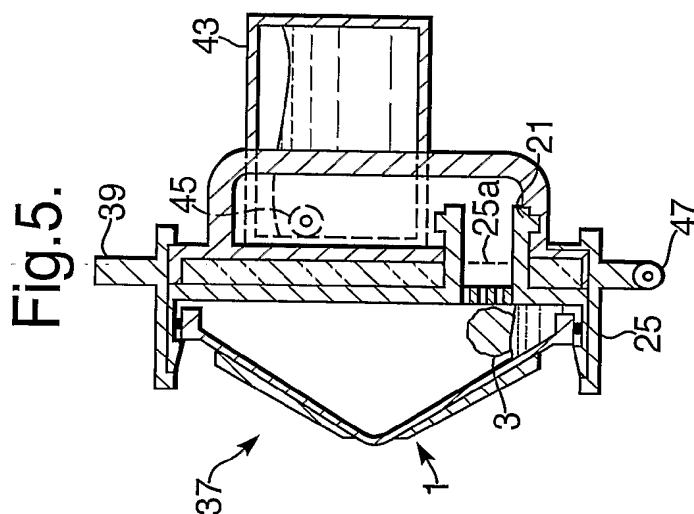


Fig.3b.



4/4



INTERNATIONAL SEARCH REPORT

International Application No
B2004/003434

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01F11/00 C12Q1/68 A61B10/00 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B01F C12Q A61B G01N A61F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6 187 546 B1 (LOKTIONOV ALEXANDRE ET AL) 13 February 2001 (2001-02-13) cited in the application column 4, line 47 - column 5, line 9 column 13	1-4
X	US 2001/002985 A1 (KLEINSASSER JONATHAN) 7 June 2001 (2001-06-07) paragraphs '0052! - '0058!; figures 6,7	5,6,8,10
X	DE 15 98 515 A (GERHARDT FABRIK UND LAGER CHEM) 27 May 1971 (1971-05-27) the whole document	5,7,10
A	US 2002/119472 A1 (LAPIDUS STANLEY N ET AL) 29 August 2002 (2002-08-29) paragraph '0038!	1-4
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

29 October 2004

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/003434

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 456 507 A (BRACONNIER THERESE) 12 December 1980 (1980-12-12)	11, 14
Y	figure 1	13
Y	DE 199 10 836 A (KERNFORSCHUNGSANLAGE JUELICH) 21 September 2000 (2000-09-21) abstract	13

INTERNATIONAL SEARCH REPORT

Information on patent family members

 International Application No
 B2004/003434

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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Application Number

*** 17/936,335 ***

Application/Control No.

17/936,335

Applicant(s)/Patent under
Reexamination

Louwagie, Joost

Examiner

WHISENANT, ETHAN C

Art Unit

1634

Document Code - DISQ

Internal Document - DO NOT MAIL

**TERMINAL
DISCLAIMER**

☒ **APPROVED**

☐ **DISAPPROVED**

Date Filed:
24 January 2023

**This patent is subject
to a Terminal
Disclaimer**

Approved/Disapproved by:

/PAMELA A YOUNG/

Technology Center: **OPLC**

Telephone: **(571)272-3622**

Patent Owner's Ex. 2004, Page 229

To: DOCKETING@CASIMIRJONES.COM, Genomic_docketing@cardinal-ip.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 173254

Jan 25, 2023 03:28:22 AM

Dear PAIR Customer:

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 173254 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
17936335	N570	01/25/2023	EXCTD-35239.306

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM



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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

173254 7590 02/16/2023
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

EXAMINER

WHISENANT, ETHAN C

ART UNIT

PAPER NUMBER

1634

DATE MAILED: 02/16/2023

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306	1513

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	05/16/2023

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.

Patent Owner's Ex. 2004, Page 231

PART B - FEE(S) TRANSMITTAL

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 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)

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 02/16/2023
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.
17/936.335	09/28/2022	Joost Louwagie	EXCTD-35239.306	1513

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	05/16/2023

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) ☐ 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. _____

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
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306	1513
173254	7590	02/16/2023	EXAMINER	
CASIMIR JONES, S.C. 2275 DEMING WAY, SUITE 310 MIDDLETON, WI 53562			WHISENANT, ETHAN C	
			ART UNIT	PAPER NUMBER
			1634	
DATE MAILED: 02/16/2023				

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.
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.

Notice of Allowability	Application No. 17/936,335	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 the paper(s) filed 23 JAN 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 1-20. 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>24 JAN 2023</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

AN 2023 NOTICE OF ALLOWABILITY

► **Claim(s) 1-20** as amended in the paper(s) filed 23 JAN 09 is/are pending in this application.

► A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed on 10 JUL 09 in this application after final rejection. 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) 27-41 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) 1-20** is/are deemed to be allowable in light of the paper(s) filed 23 JAN 2023 and the persuasive argument(s) therein.

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, Dave Nguyen, can be reached at (571) 272-0731.

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

Issue Classification 	Application/Control No. 17/936,335	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: 20	
/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. 17/936,335	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			
/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

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

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 20	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner) _____ (Date) _____		02 February 2023 (Date)	O.G. Print Claim(s) 1 O.G. Print Figure None

Issue Classification 	Application/Control No. 17/936,335	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
	1		10		19										
	2		11		20										
	3		12												
	4		13												
	5		14												
	6		15												
	7		16												
	8		17												
	9		18												

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 20					
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner) _____ (Date) _____		02 February 2023 (Date)	<table border="1"> <tr> <td>O.G. Print Claim(s)</td> <td>O.G. Print Figure</td> </tr> <tr> <td>1</td> <td>None</td> </tr> </table>	O.G. Print Claim(s)	O.G. Print Figure	1	None
O.G. Print Claim(s)	O.G. Print Figure						
1	None						

<i>Index of Claims</i> 	Application/Control No. 17/936,335	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

[illegible]

Search Notes 	Application/Control No. 17/936,335	Applicant(s)/Patent Under Reexamination Louwagie, Joost
	Examiner Ethan C Whisenant	Art Unit 1634

CPC - Searched*		
Symbol	Date	Examiner
C12Q 1/68 ; 2600/154;and 2600/158	12/30/2022	ECW
G01N 33/57446; 2333/805	12/30/2022	ECW
Updated Search	02/02/2023	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.

Search Notes		
Search Notes	Date	Examiner
See the Examiner's Search Notes on the last pg of the OA with paper no.20221219	12/30/2022	ECW
Updated Search	02/02/2023	ECW

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
C12Q	1/68 ; 1/6886	02/02/2023	ECW
G01N	33/574	02/02/2023	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	
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Patent Owner's Ex. 2004, Page 242

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	17936335
				Filing Date	09/28/2022
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT
Sheet	1	of	2	Attorney Docket Number	EXCTD-35239.306

U.S. PATENTS					
Exami ner Initials*	Cite No. ¹	Document Number	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
		Number-Kind Code ² (if known)			
		6761702	2004-07-13	SMITH	

U.S. PUBLISHED PATENT APPLICATIONS					
Exami ner Initials*	Cite No. ³	Document Number	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
		Number-Kind Code ⁴ (if known)			
		20020096469	2002-07-25	FAULKNER	
		20050244836	2005-11-03	TSANG	
		20070072214	2007-03-29	GARVIN	

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	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
		Country Code ⁵ Number ⁶ Kind Code ⁷ (if known)				
		WO 2000/050640	2000-08-31	EXACT LABORATORIES, INC.		
		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.	Translatio n ⁶

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	01/31/2023
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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.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /E.C.W/

Patent Owner's Ex. 2004, Page 243

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				<i>Application Number</i>	17936335
				<i>Filing Date</i>	09/28/2022
				<i>First Named Inventor</i>	JOOST LOUWAGIE
				<i>Art Unit</i>	1634
				<i>Examiner Name</i>	WHISENANT
Sheet	2	of	2	<i>Attorney Docket Number</i>	EXCTD-35239.306

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)	2023-01-24
Name/Print	Mary Ann D. Brow	Registration Number	42363

Patent Owner's Ex. 2004, Page 244

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.

³ Applicant's unique citation designation number (optional).

⁴ See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04.

⁵ Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3).

⁶ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document.

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

⁸ Applicant is to place a check mark here if English language Translation is attached.

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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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306

CONFIRMATION NO. 1513

PUBLICATION NOTICE

173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562



OC000000137413827

Title:FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Publication No.US-2023-0048152-A1

Publication Date:02/16/2023

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently <http://www.uspto.gov/patft/>.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently <https://portal.uspto.gov/pair/PublicPair>. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Patent Owner's Ex. 2004, Page 246

To: DOCKETING@CASIMIRJONES.COM, Genomic_docketing@cardinal-ip.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 173254

Feb 23, 2023 12:34:20 PM

Dear PAIR Customer:

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 173254 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

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Application	Document	Mailroom Date	Attorney Docket No.
17936335	NOA	02/16/2023	EXCTD-35239.306
	1449	02/16/2023	EXCTD-35239.306
	NTC.PUB	02/16/2023	EXCTD-35239.306

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MIDDLETON, WI 53562



**Courtesy Reminder for
Application Serial No: 17/936,335**

Attorney Docket No: EXCTD-35239.306

Customer Number: 173254

Date of Electronic Notification: 02/23/2023

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Joost Louwagie	Confirmation: 1513
Serial No.: 17/936,335	Group No.: 1634
Filed: 09/28/2022	Examiner: Ethan C. Whisenant
Entitled: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

**COMMENTS REGARDING EXAMINER'S STATEMENT ON
REASONS FOR ALLOWANCE MAILED 02/16/2023**

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 02/16/2023 for the above-referenced application. Applicant agrees with the Examiner's conclusion that claims 1-20 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 Allowability in the above-referenced application includes the text reproduced below:

AN 2023 NOTICE OF ALLOWABILITY

- ▶ **Claim(s) 1-20** as amended in the paper(s) filed 23 JAN 09 is/are pending in this application.
- ▶ A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed on 10 JUL 09 in this application after final rejection. 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) 27-41 is/are now pending.

For at least the reasons set forth below, it is clear that both of the paragraphs reproduced above do not pertain to the above-referenced application, and were erroneously included in the Examiner's statement:

- The instant application was not pending in 2009, the year referenced for filing of papers in both of the reproduced paragraphs;

- The 1st reproduced paragraph references claims “as amended” but the allowed claims of have not been amended since the filing date of the instant application;
- A Request for Continued Examination, referenced in the 2nd reproduced paragraph, has never been filed in this application; and
- Claims 27-41, referenced in the 2nd reproduced paragraph, have never been presented in this application.

It is Applicant’s position that the text in the paragraphs reproduced above was clearly included in the Examiner’s statement of Reasons for Allowability by clerical error. It is further Applicant’s position that the Examiner’s statement as a whole, including the text included by error, has no bearing on the allowability, scope, interpretation, or any other aspect of any of allowed claims 1-20 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: March 9, 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

Patent Owner's Ex. 2004, Page 2502 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
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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.

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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)
03/09/2023	(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
17/936,335	09/28/2022	LOUWAGIE, Joost	EXCTD-35239.306	1513

TITLE OF INVENTION:

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	05/16/2023

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/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

(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) ☐ 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 03/09/2023

Typed or printed name Mary Ann D. Brow

Registration No. 42363

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.

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

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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.
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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 ACKNOWLEDGEMENT RECEIPT

APPLICATION #
17/936,335

RECEIPT DATE / TIME
03/09/2023 01:39:03 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 # 1513

FILED BY Stephanie Filandrinos

PATENT CENTER # 61715194

FILING DATE 09/28/2022

CUSTOMER # 173254

FIRST NAMED
INVENTOR Joost Louwagie

CORRESPONDENCE
ADDRESS -

AUTHORIZED BY Mary Ann Brow

Documents

TOTAL DOCUMENTS: 2

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-03-09_IssueFeeTransmittal_EXC TD-003USCON3.35239_35239.3 06.pdf	2	Issue Fee Payment (PTO-85B)	450 KB
Warning: The attached file contains comments or annotations. Comments are not recommended and may cause processing problems with the document.			
2023-03-09_CommentsExaminersStat ement_EXCTD-003USCON3- 35239_35239-306.pdf	2	Request for Corrected Notice of Allowance	123 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2023-03-09_IssueFeeTransmittal_EXCTD-003USCON3.35239_35239.306.pdf	43507A9A9DC0C18D9FD018A1290493682E8290579ADFF78BB 463AA6E2BEF2F872C59AB42A7FD0727918BDA49C7F5133A4C D5D1DCA9613C857FCCBCD2B77E44B5
2023-03-09_CommentsExaminersState ment_EXCTD-003USCON3-35239_35239-306.pdf	912626D480DCB2512E18FC6578DD11B2017C0DCD537AF078B 21A00F4BBE1E5525D44D838091DE7A5A2B8E0C1258741642E 27AAD763CC0AC19AE0526F1451FD2E

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New Applications Under 35 U.S.C. 111

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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.

ELECTRONIC PAYMENT RECEIPT

APPLICATION #
17/936,335

RECEIPT DATE / TIME
03/09/2023 01:39:03 PM ET

ATTORNEY DOCKET #
EXCTD-35239.306

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 # 1513

FILED BY Stephanie Filandrinos

PATENT CENTER # 61715194

AUTHORIZED BY Mary Ann Brow

CUSTOMER # 173254

FILING DATE 09/28/2022

CORRESPONDENCE
ADDRESS -

FIRST NAMED
INVENTOR Joost Louwagie

Payment Information

PAYMENT METHOD
CARD / 0638

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E202339D39576209

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Stephanie Filandrinos

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Patent Owner's Ex. 2004, Page 256

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i>				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	2	of	5	Attorney Docket Number	EXCTD-35239.306

U.S. PUBLISHED PATENT APPLICATIONS					
		20080124714	2008-05-29	SHUBER et al.	
		20080221056	2008-09-11	BAYLIN et al.	
		20080227208	2008-09-18	YEE et al.	
		20090004058	2009-01-01	LIANG et al.	
		20100136572	2010-06-03	ATAMAN-ONAL et al.	
		20120164238	2012-06-28	JOOST	
		20160194723	2016-07-07	JOOST	

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

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translation ⁸
		Country Code ⁵ Number ⁶ Kind Code ⁷ (if known)				
		EP 0032782	1981-07-29	KOMMANDIITTIYHTIO FINNPIPETTE		
		EP 0308227	1989-03-22	SMITHKLINE DIAGNOSTICS, INC.		
		EP 0817968	1998-01-14	EXACT SCIENCES CORPORATION		
		FR 2919065	2009-01-23	BIOMERIEUX		
		WO 1990/06995	1990-06-28	SISKA DIAGNOSITCS, INC.		
		WO 1997/46705	1997-12-11	THE JOHNS HOPKINS SCHOOL OF MEDICINE		
		WO 2004/067726	2004-08-12	KECK GRADUATE INSTITUTE		
		WO 2004/083399	09/2004			
		WO 2004/0927209	2004-10-28	KIETURAKIS & KARCZEWSKI		
		WO 2005/017207	2005-02-04	CASE WESTERN RESERVE UNIVERSITY		
		WO 2006/113671	2006-10-26	ONCOMETHYLOME SCIENCES SA		

Change(s) applied
to document,
/S.J.W/
3/9/2023

Examiner Signature		Date Considered	
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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.



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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/936,335	04/25/2023	11634781	EXCTD-35239.306	1513

173254 7590 04/05/2023
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
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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The Patent Term Adjustment is 0 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.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

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INVENTOR(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional inventors):

Joost Louwagie, Dornach, SWITZERLAND;

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Exact Sciences Corporation, Madison, WI;

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17936335	ISSUE.NTF	04/05/2023	EXCTD-35239.306

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/936,335	09/28/2022	Joost Louwagie	EXCTD-35239.306	1513
173254	7590	04/27/2023	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.
17/936,335	25-APR-23	11634781

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
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Application	Document	Mailroom Date	Attorney Docket No.
17936335	M327	04/27/2023	EXCTD-35239.306

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