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January 05, 2024

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PATENT NUMBER: ***11845991***
ISSUE DATE: ***December 19, 2023***

Certified by

Performing the Functions and Duties of the
Under Secretary of Commerce
for Intellectual Property
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Application Number: 15634607 Document Date: 06/27/2017

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PTO/AIA/14 (11-15)
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	

The application data sheet is part of the provisional or nonprovisional 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.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 — Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Joost		Louwagie	

Residence Information (Select One) US Residency ● Non US Residency Active US Military Service

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State/Province	WI
Postal Code	53719
Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	72960		
Email Address	docketing@casimirjones.com	Add Email	Remove Email

Application Information:

Title of the Invention	METHODS FOR DETECTING COLORECTAL CANCER		
Attorney Docket Number	EXCT-35239/US-4/CON	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)		**Suggested Figure for Publication (if any)**	

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Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

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).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	◯ Limited Recognition (37 CFR 11.9)
Customer Number	72960		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 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.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	15010436	2016-01-29

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Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
15010436	Continuation of	13147570	2012-03-12
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13147570	a 371 of international	PCT/GB2010/000180	2010-02-03
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/GB2010/000180	Claims benefit of provisional	61149581	2009-02-03

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. 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)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(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).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ 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.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed 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)

A. 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).

B. 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).

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 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.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	

Applicant Information:

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.

Applicant 1 — Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. 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. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Exact Sciences Corporation		
Mailing Address Information For Applicant:			
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Address 2			
City	Madison	**State/Province**	WI
Country	US	Postal Code	53719
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

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.

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Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

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

Signature	/Mary Ann D. Brow/			Date (YYYY-MM-DD)	2017-06-27
First Name	Mary Ann D.	Last Name	Brow	Registration Number	42363

Additional Signature may be generated within this form by selecting the Add button. Add

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	

This collection of information is required by 37 CFR 1.76. 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 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet 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 CooperationTreaty.

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.

METHODS FOR DETECTING COLORECTAL CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

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

FIELD OF THE INVENTION

The present invention is concerned with the diagnosis, staging and treatment of disease, in particular cancer and more specifically colorectal cancer. The invention relates to methods and kits for diagnosing colorectal cancer based upon detecting epigenetic modifications, typically in specific genes. The methods and kits may also permit the detection of blood in a fecal sample, with the combined tests proving particularly advantageous.

BACKGROUND OF THE INVENTION

Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide, and is 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 perioxidase 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 protpoporphyrin 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 feacal 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 feacal 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.

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.

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.

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.

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

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.

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.

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).

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.

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,

(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.

a method for predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethyiating 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 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.

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.

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

(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.

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:

(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.

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

(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.

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 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; 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 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.

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 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 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 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 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 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 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 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 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 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. 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 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 (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.

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.

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.

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 metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase).

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.

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.

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 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 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 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 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 fojlow 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 -2O0C 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 a!., 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 diferences 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 un methylated cytosiπe 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, SJ. (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 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 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 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 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 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. 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.

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 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®, MOLECULAR BEACONS®, AMPLIFLUOR® and SCORPION® DzyNA®, PlexorTM 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.

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 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.

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 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 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 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 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 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 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 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 decribed 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 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 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 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 mimimal 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.

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 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 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 (FOXE1), and 7 and 8 (NDRG4). In further 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 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 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 (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).

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 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. 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, 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 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, 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.

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.

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 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 ,

(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.

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 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.

Furthemore, 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 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.

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 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 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 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 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, 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 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-fluouro-2'-deoxycytidine, pseudoisocytidine, 5,6-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 CDOl 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 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 (FOXE1), and 9 (NDRG4).

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 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 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 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 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 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. 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/purificatton of DNA. 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 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 to be detected as an indication of the methtylation 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:

(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 (FOXE1), 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 (FOXE1), 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 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 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 methtylation 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.

EXPERIMENTAL SECTION

Example 1

Sample collection and processing

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 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.

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 5 minutes at 2540 rcf at 200C. 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 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 matrix. The supernatant was transferred into a new microcentrifuge tube. 25 µl of proteinase K was added to 60OuI 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 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 µl proteinase K to 2 ml of the supernatant, mix well, add 2.4 ml of AL Buffer and vortex for 15 s. Incubate at 7O0C 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 µl 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 200µl 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 370C for 15 minutes shaking at 1100 rpm. Then 100 µl of the diluted CT Conversion Reagent was added and samples were incubated at 7O0C 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-HCI 1mM pH8.0 and stored at -800C, 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 5OuI) containing buffer (16.6mM (NH4)2SO4, 67 mM Tris (pH 8.8), 6.7 mM MgCI2, 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' - TTATTTTGCGAGCGGTTTC - 3	1
	reverse primer	5' - GAATACTCTAATTCCACGCGACT - 3'	2
	beacon	5'-FAM- CGACATGCGGGTTCGGTCGGCGCGGGGCATGTCG-3'-DABCYL	3
FOXE1	forward primer	5' - TTTGTTCGTTTTTCGATTGTTC - 3'	4
	reverse primer	5' - TAACGCTATAAAACTCCTACCGC - 3'	5
	beacon	5'-FAM- CGTCTCGTCGGGGTTCGGGCGTATTTTTTTAGGTAGGCGAGACG -3'-DABCYL	6
NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'	7
	reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 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-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-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'-GTTGGGTTTTCGTAGTTTTGTAGATCGC-3'	16
	Reverse primer	5'-CTACGCCCAAACTCGACG-3'	17
	beacon	5'-FAM-CGACATGCCCCGCCCTATCGCCGAAATCGCATGTCG-DABCYL-3'	18
SFRP2	Forward primer	5' – GGGTCGGAGTTTTTCGGAGTTGCGC – 3'	19
	Reverse primer	5' – CCGCTCTCTTCGCTAAATACGACTCG – 3'	20
	beacon	5'-FAM- CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTCGGGCATGTCG - 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-CGACATGCGTTCGTGTTTTGGTTTGTCGCGGTTTGGCATGTCG DABCYL-3'	27
LAMA1	Forward primer	5'-TTTTTAGATTTATCGAGTGGCG-3'	28
	Reverse primer	5'-CGAACTCACCTCTCTACCGAC-3'	29
	beacon	5'-CGACATGCCAAAAACACGCCCCCGCGCATGTCG-3'	30

MMP2	Forward primer	5'-TTCGGGTTATTAGCGTTTTTATC-3'	31
	Reverse primer	5'-ACTCCAACCAAACGACGAA-3'	32
	beacon	5'-FAM-CGACATCGTTGGTTCGGTGCGTGTGGTCGATGTCG-DABCYL-3'	33
CDO1	Forward primer	5'-AATTTGATTTGTGTGTGTATCGC-3'	34
	Reverse primer	5'-GAAACGTAAAAATATCGTCGCA-3'	35
	beacon	5'-FAM-CGACATGCGCGATTTCGGATTTATTGCGTTGTTAGGGCATGTCG-DABCYL-3'	36
TFPI2	Forward primer	5'-GTTCGTTGGGTAAGGCGTTC-3'	37
	Reverse primer	5'-CATAAAACGAACACCCGAACCG-3'	38
	Beacon	5'-FAM-CGACATGCACCGCGCACCTCCTCCCGCCAAGCATGTCG-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. 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%.

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.

		Methylation marker only		Methylation markers plus FIT (100 ng/ml cutoff)	
Gene	cutoffs (copies)	Spec (n=21)	Sens (n=23)	Spec (n=21)	Sens (n=23)
GATA4	10	90%	52%	86%	87%
GPNMB	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)	96%	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%
Sensivity for advanced adenoma	0%	0%	10%	10%	20%	10%	10%
Sensivity 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%
Sensivity for advanced adenoma	10%
Sensivity 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 -200C until further use.

In this study only a portion of the stool sample (« 1g) was investigated for further methylation testing. First, historical samples were thawed at the storage site and partial samples were taken (= "Ig) using special designed stool tubes with integrated measuring spoons (Sarstedt). Each sample was then weighted and 2 volumes of stabilizing buffer (Diagn MoI Pathol; Volume 14, Number 3, September 2005) were added. Samples were stored at -800C 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 250µl of homogenized stool material each, and elute in 75µl of buffer 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

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 2µg of input DNA was used and elution was done in 30µl Tris-HCI (1mM, pH 8.0); samples were stored at -8O0C, 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 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 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 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.

2. The method of claim 1 wherein detecting the presence of blood in the faecal sample comprises detection of haemoglobin in the faecal sample.

3. The method of claim 2 wherein haemoglobin in the faecal sample is detected through immunochemical means.

4. The method of claim 2 or 3 wherein the result in step (a) is considered positive if the concentration of hemoglobin detected is at least between about 50 to 150 ng/ml.

5. The method of claim 2 or 3 wherein the result in step (a) is considered positive if the concentration of hemoglobin detected is at least about 100 ng/ml.

6. The method of claim 2 or 3 wherein the result in step (a) is considered positive if the concentration of hemoglobin detected is greater than a predetermined threshold level, which threshold level is lower than is typically employed as the threshold concentration of hemoglobin in hemoglobin detection tests, but for those samples in which a lower concentration of hemoglobin is detected, which would be considered a negative result in typically employed tests, step (b) is performed only on these samples and the detection of the epigenetic modification in step (b) is then used to confirm the positive result in step (a).

7. The method of claim 2 or 3 wherein the result in step (a) is considered positive if the concentration of hemoglobin detected is at least about 10 ng/ml and wherein step (b) is performed only in the event that the concentration of hemoglobin detected is between about 10 ng/ml and about 200 ng/ml and the detection of the epigenetic modification in step (b) is then used to confirm the positive result in step (a).

8. The method of any preceding claim wherein 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 , 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.

9. The method of any preceding claim wherein the epigenetic modification is detected in at least one gene 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.

10. The method of claim 9 wherein 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.

11. The method of claim 10 wherein the panel of genes comprises, consists essentially of or consists of PHACTR3, NDRG4 and FOXE1, NDRG4 and FOXE1 , PHACTR3 and NDRG4, or PHACTR3 and FOXE1.

12. The method of any preceding claim wherein the epigenetic modification is methylation.

13. The method of any preceding claim which utilises primers selected from primers comprising the nucleotide sequences set forth in table 1 , in particular which utilises primers selected from primers comprising 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.

14. The method of any preceding claim which utilises probes selected from probes comprising the nucleotide sequences set forth in table 1 , in particular which utilises probes selected from probes comprising 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.

15. The method of any preceding claim wherein a single faecal sample is utilised as the source of the sample for (a) and (b).

16. The method of claim 15 wherein the faecal sample is split to permit (a) and (b) to be carried out.

17. A method of sample processing, prior to carrying out a method of any of claims 1 to 16, 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.

18. The method of claim 17 which further comprises forwarding or otherwise delivering the remaining portion of the collected faecal sample to a laboratory for performing step (b) of the method of claim 1, optionally as further defined in any one of claims 8 to 14, on the remaining portion of the collected faecal sample.

19. The method of claims 17 or 18 which further comprises forwarding or otherwise delivering the removed portion of the collected faecal sample to a laboratory for performing step (a) of the method of claim 1, optionally as further defined in any of claims 2 to 7, on the removed portion of the sample.

20. The method of any of claims 17 to 19 which further comprises performing step (b) of the method of claim 1 , optionally as further defined in any one of claims 8 to 14, on the remaining portion of the collected faecal sample.

21. The method of any of claims 17 to 20 which further comprises performing step (a) of the method of claim 1 , optionally as further defined in any of claims 2 or 7, on the removed portion of the sample.

22. The method of any preceding claim wherein the faecal sample utilised to perform step (b) weighs no more than approximately 4 grams.

23. 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.

24. 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 panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 in a sample, wherein detection of a reduction in the epigenetic modification in at least one of the genes in the panel as treatment progresses is indicative of successful treatment.

25. 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 panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 in a sample, wherein detection of the epigenetic modification in at least one of the genes in the panel is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

26. 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 panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 in a sample, wherein detection of the epigenetic modification in at least one of the genes in the panel is indicative that the likelihood of resistance to treatment is lower than if the epigenetic modification is not detected.

27. A method of selecting a suitable treatment regimen for colorectal cancer comprising detecting an epigenetic modification in a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1 in a sample, wherein detection of the epigenetic modification in at least one of the genes in the panel 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.

28. The method of any of claims 23 to 27 wherein the epigenetic modification is methylation and thus the method involves determining the methylation status of the panel of genes.

29. The method of any of claims 23 to 28 which utilises primers selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOXE1), and 7 and 8 (NDRG4).

30. The method of any of claims 23 to 29 which utilises probes selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOXE1), and 9 (NDRG4).

31. The method of any of claims 23 to 30 wherein the sample is a faecal sample.

32. The method of claim 31 wherein the faecal sample weighs no more than approximately 4 grams.

33. 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 as claimed in any of claims 23 to 32.

34. A method of detecting a predisposition to, or the incidence of, 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.

35. A method for monitoring treatment of cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1 in a sample, wherein detection of a reduction in the epigenetic modification in the at least one gene as treatment progresses is indicative of successful treatment.

36. A method for predicting the likelihood of successful treatment of cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1 in a sample, wherein detection of the epigenetic modification in the at least one gene is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

37. A method for predicting the likelihood of resistance to treatment of cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1 in a sample, wherein detection of the epigenetic modification 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.

38. A method of selecting a suitable treatment regimen for cancer comprising detecting an epigenetic modification in at least one gene selected from LAMA1 and CDO1 in a sample, wherein detection of the epigenetic modification 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.

39. The method of any of claims 34 to 38 wherein the cancer is colorectal cancer.

40. The method of any of claims 34 to 39 wherein the epigenetic modification is methylation and thus the method involves determining the methylation status of the at least one gene.

41. The method of any of claims 34 to 40 which utilises primers selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 28 and 29 (LAMA1) and 34 and 35 (CDO1).

42. The method of any of claims 34 to 41 which utilises probes selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 30 (LAMA1) and 36 (CDO1).

43. The method of any of claims 34 to 42 wherein the sample is a faecal sample.

44. The method of claim 43 wherein the faecal sample weighs no more than approximately 4 grams.

45. 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 as claimed in any of claims 34 to 44.

46. A method of detecting a predisposition to, or the incidence of, colorectal cancer 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.

47. 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 at least one gene selected from GPNMB and MMP2 in a faecal sample, wherein detection of a reduction in the epigenetic modification in the at least one gene as treatment progresses is indicative of successful treatment.

48. 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 at least one gene selected from GPNMB and

MMP2 in a faecal sample, wherein detection of the epigenetic modification in the at least one gene is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

49. 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 at least one gene selected from GPNMB and MMP2 in a faecal sample, wherein detection of the epigenetic modification 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.

50. A method of selecting a suitable treatment regimen for colorectal cancer comprising detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2 in a faecal sample, wherein detection of the epigenetic modification 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.

51. The method of any of claims 46 to 50 wherein the epigenetic modification is methylation and thus the method involves determining the methylation status of the at least one gene.

52. The method of any of claims 46 to 51 which utilises primers selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 22 and 23 (GPNMB) and 31 and 32 (MMP2).

53. The method of any of claims 46 to 52 which utilises probes selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 24 (GPNMB) and 33 (MMP2).

54. 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 as claimed in any of claims 46 to 53.

55. 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.

56. The kit of claim 55 wherein 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.

57. The kit of claim 55 or 56 wherein the epigenetic modification is detected in at least one gene selected from PHACTR3, NDRG4 and FOXE1.

58. The kit of claim 57 wherein 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.

59. The kit of any one of claims 55 to 58 wherein the means for detecting an epigenetic modification in the DNA contained within the faecal sample comprise primers and/or probes which permit the methylation status of the DNA to be determined directly.

60. The kit of claim 59 wherein the primers are selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOXE1), and 7 and 8 (NDRG4).

61. The kit of claim 59 or 60 wherein the probes are selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOXE1), and 9 (NDRG4).

62. The kit of any of claims 55 to 61 wherein the means for detecting the presence of blood in the faecal sample comprises means for detection of haemoglobin in the faecal sample.

63. The kit of claim 62 wherein haemoglobin in the faecal sample is detected through immunochemical means.

64. 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 a panel of at least two genes selected from PHACTR3, NDRG4 and FOXE1.

65. The kit of claim 64 wherein 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.

66. The kit of claim 65 wherein the primers are selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 1 and 2 (PHACTR3), 4 and 5 (FOXE1), and 7 and 8 (NDRG4).

67. The kit of claim 65 or 66 wherein the probes are selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 3 (PHACTR3), 6 (FOXE1), and 9 (NDRG4).

68. 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

69. The kit of any of claims 55 to 68 which further comprises means for processing a faecal sample.

70. The kit of claim 68 or 69 wherein 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 DNA to be determined directly.

71. The kit of any of claims 68 to 70 wherein the primers are selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 28 and 29 (LAMA1) and 34 and 35 (CDO1).

72. The kit of any of claims 68 to 71 wherein the probes are selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 30 (LAMA1) and 36 (CDO1).

73. 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.

74. The kit of claim 73 wherein the means for detecting an epigenetic modification in at least one gene selected from GPNMB and MMP2 comprise primers and/or probes which permit the methylation status of the DNA to be determined directly.

75. The kit of claim 73 wherein the primers are selected from primers comprising the nucleotide sequences set forth as SEQ ID Nos: 22 and 23 (GPNMB) and 31 and 32 (MMP2).

76. The kit of claim 73 or 74 wherein the probes are selected from probes comprising the nucleotide sequences set forth as SEQ ID Nos: 24 (GPNMB) and 33 (MMP2).

77. The kit of any of claims 69 to 76 wherein the means for processing a faecal sample comprises a sealable vessel for collection of a faecal sample.

78. The kit of any of claims 55 to 77 comprising a container for a portion of the collected faecal sample suitably dimensioned to contain a faecal sample weighing no more than approximately 4 grams.

79. The kit of claim 78 further comprising a collection device adapted to retrieve and/or deposit the faecal sample in the container.

80. The kit of any of claims 55 to 79 which further comprises 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.

81. A method of detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising 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.

ABSTRACT

A method of detecting a predisposition to, or the incidence of, colorectal cancer in a fecal sample comprises, in a first step (a), detecting the presence of blood in the fecal sample, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer. The method additionally comprises, in second step (b), detecting an epigenetic modification in the DNA contained within the fecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer. Based upon a positive result obtained in either (a) or (b) or in both (a) and (b) a predisposition to, or the incidence of, colorectal cancer is detected. Related methods and kits involve detecting an epigenetic modification in a number of specific genes.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Filer:	Mary Ann Deese Brow
Attorney Docket Number:	EXCT-35239/US-4/CON

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	280	280
UTILITY SEARCH FEE	1111	1	600	600
UTILITY EXAMINATION FEE	1311	1	720	720
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1600**

Electronic Acknowledgement Receipt

EFS ID:	29616979
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCT-35239/US-4/CON
Receipt Date:	27-JUN-2017
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Time Stamp:	16:22:05
Application Type:	Utility under 35 USC 111(a)

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Payment Type	DA
Payment was successfully received in RAM	$1600
RAM confirmation Number	062817INTEFSW00003505504302
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	35239US4CON_ADS.pdf	1823374 9f62d792c2f6b2961454805db92355c75e760528	no	8
Warnings:					
Information:					
2		35239US4CON_Application.pdf	543372 37d53df814fe29825e7e4e1227d6871814093531	yes	68

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	55
Claims	56	67
Abstract	68	68

Warnings:					
Information:					
3	Sequence Listing (Text File)	35239-US-4-CON.txt	7348	no	-
Warnings:					
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4	Fee Worksheet (SB06)	fee-info.pdf	35009 11fc830987d4edbd7ccc29d7dbbd956a0a04a455	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	2409103		

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If a new application is being filed and the application includes the necessary components for a 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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If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
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Validated By CRFValidator v 1.0.5

Application No: 15634607 Version No: 1.0

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Total Warnings: 39
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Error code	Error Description
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SEQUENCE LISTING

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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
15/634,607	06/27/2017	Joost Louwagie	EXCT-35239/US-4/CON

CONFIRMATION NO. 1874

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

FORMALITIES LETTER

OC000000092734943

Date Mailed: 07/13/2017

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing.

Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- Additional claim fees of $ **14820** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **140** surcharge.
- $ **7560** for **18** independent claims over 3.
- $ **6480** for **81** total claims over 20.
- $ **780** for multiple dependent claim surcharge.
- $(**0**) previous unapplied payment amount.
- $ **14960** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Joost Louwagie

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". 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 you are not using EFS-Web to submit your reply, you must include a copy of this notice.

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**.

/hchin/

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
15634607	

Applicant(s) Joost Louwagie

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
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Total Indep	21		0		0	
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Total Claims	101		0		0	

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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	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/634,607	06/27/2017	1797	1600	EXCT-35239/US-4/CON	81	21

CONFIRMATION NO. 1874

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

FILING RECEIPT

OC000000092734942

Date Mailed: 07/13/2017

Receipt is acknowledged of this non-provisional 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 APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

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 15/010,436 01/29/2016
which is a CON of 13/147,570 03/12/2012
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: 07/12/2017

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/634,607**

Projected Publication Date: 10/19/2017

Non-Publication Request: No

Early Publication Request: No

Title

METHODS FOR DETECTING COLORECTAL CANCER

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).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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 AssetsControl, 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.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/634,607

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	101 minus 20 =	* 81			OR	x 80 =	6480
INDEPENDENT CLAIMS (37 CFR 1.16(h))	21 minus 3 =	* 18				x 420 =	7560
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).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							780
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	16420

APPLICATION AS AMENDED - PART II

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

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

To: docketing@casimirjones.com,pto.correspondence@casimirjones.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 72960

Jul 13, 2017 08:45:56 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, 72960 , 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.
15634607	NTC.MISS.PRT	07/13/2017	EXCT-35239/US-4/CON
	APP.FILE.REC	07/13/2017	EXCT-35239/US-4/CON

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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:

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PATENT
Attorney Docket No.: **EXCTD-35239/US-4/CON**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	1874
Serial No.:	15/634,607	Group No.:	Not yet assigned
Filed:	06/27/2017	Examiner:	Not yet assigned
Entitled:	**METHODS FOR DETECTING COLORECTAL CANCER**		

AMENDMENT AND RESPONSE TO NOTICE TO FILE MISSING PARTS MAILED 07/13/2017

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

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

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

Dated: October 13, 2017 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This paper is responsive to the Notice to File Missing Parts mailed 07/13/2017, with response due by 09/13/2017. A one month extension of time is requested. Prior to the Examination of the present Application, Applicants request the following amendments be entered.

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/US-4/CON**. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Amendments to the Specification begin on Page 2;

Amendments to the Claims are reflected in the Listing of Claims beginning on Page 3;

Remarks begin on Page 6.

IN THE SPECIFICATION:

Please replace the Specification with the Substitute Specification provided herewith.

AMENDMENTS TO THE CLAIMS:

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

1-81. (cancelled)

82. (new) A method of processing a fecal sample, the method comprising

a) collecting a fecal sample from a human subject;

b) removing a portion of said fecal sample to produce a removed portion and a remaining portion of said fecal sample;

c) combining the removed portion with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample;

d) stabilizing the remaining portion of the fecal sample.

83. (new) The method of claim 82, wherein said stabilizing of said remaining portion of the fecal sample comprises one or both of:

i) adding a stabilizing buffer to said remaining sample;

ii) freezing said remaining sample.

84. (new) The method of claim 82, further comprising delivering the removed portion and the remaining portion of the fecal sample to a medical diagnostics laboratory.

85. (new) A set of articles for collection of a fecal sample at home by a human subject, comprising:

a) a sealable vessel for collection of a fecal sample, said sealable vessel configured for a human subject to directly defecate into the sealable vessel;

b) a container for receiving a removed portion of a fecal sample;

c) a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample from a human subject;

d) a stabilizing buffer for stabilizing a remaining portion of a fecal sample.

86. (new) The set of articles of claim 85, wherein said buffer that prevents denaturation or degradation of blood proteins found in a fecal sample from a human subject is contained in said container for receiving a removed portion of a fecal sample.

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

A) obtaining a pair of portions of a fecal sample from a human subject, the pair of portions comprising a removed portion and a stabilized remaining portion of a fecal sample obtained by the method of claim 82, and

B) testing the removed portion of said fecal sample for the presence of blood.

88. (new) The method of claim 87, wherein said testing comprises testing the removed portion of said fecal sample for a concentration of hemoglobin present in said removed portion.

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

90. (new) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml.

91. (new) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml.

92. (new) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml.

93. (new) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin

detected in said removed portion is at least 50 ng/ml.

94. (new) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml.

95. (new) The method of claim 87, further comprising

C) extracting DNA from the stabilized remaining portion of said fecal sample; and

D) analyzing said DNA for the presence of DNA from a human, wherein the DNA from a human has an epigenetic modification.

96. (new) The method of claim 95, wherein said epigenetic modification comprises aberrant methylation.

97. (new) The method of claim 95, wherein said aberrant methylation comprises hypermethylation.

98. (new) The method of claim 95, wherein said DNA from a human comprises a gene and/or a promoter region of a gene.

99. (new) The method of claim 98, wherein said gene selected from the group consisting of PHACTR3, NDRG4, FOXE1 , GATA4, GPNMB, TFPI2, SOX17, SYNE1 , LAMA, MMP2, OSMR, SFRP2, and CDO1.

100. (new) The method of claim 96, wherein analyzing said DNA comprises modifying said DNA with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.

REMARKS

The Specification is herein amended by providing a Substitute Specification in clean and marked-up forms. Amendment is made to the Title, Field of the Invention, and the Abstract, and to correct typographical errors in the body of the Specification.

The amendments to the Title, Field, and Abstract reflect the subject matter of new claims 82-100 discussed below, and find support throughout the Specification and claims as originally filed, and as set forth below. These amendments do not add new matter.

Claims 1-81 are cancelled by the present amendment and new claims 82-100 are provided. Support for the new claims is found in the claims as filed, and further in the Specification as originally filed, as follows:

Support for claim 82 is provided, *e.g.*, at page 28, second full paragraph.

Support for claim 83 is provided *e.g.*, at page 28, second full paragraph; and the paragraph bridging pages 18-19.

Support for claim 84 is provided *e.g.*, in the paragraph bridging pages 28-29, and in the first full paragraph on page 29.

Support for claims 85 and 86, is provided, *e.g.*, at page 28, second full paragraph.

Support for claims 87-89 is provided, *e.g.*, at page 12, first full paragraph.

Support for claims 90-94 is provided, *e.g.*, in the last paragraph on page 13.

Support for claim 95 is provided, *e.g.*, at page 28, second full paragraph, and page 19, first full paragraph.

Support for claims 96-98 is provided, *e.g.*, at page 20, third paragraph.

Support for claims 99-100 is provided, *e.g.*, at page 37, third paragraph.

Support for claim 100 is provided, *e.g.*, at page 23, first paragraph.

These amendments do not introduce new matter.

In the Notice of Missing Parts mailed on 07/13/2017, the Office indicted that the following fees are due:

- $ **140** surcharge.
- $ **7560** for18 independent claims over 3.
- $ **8480** for 81 total claims over 20.
- $ **780** for multiple dependent claim surcharge.

The Application as amended herein contains 0 claims over 20 and 0 independent claims over 3. As such, no fees for excess claims are believed due. The following fees are paid herewith:

- $ **140** surcharge.

Applicant believes no additional fees are due.

If the Examiner wishes to discuss this case, Applicant encourages the Examiner to call the undersigned at 608-662-1277 at the Examiner's convenience.

Dated: October 13, 2017

/Mary Ann D. Brow/
Mary Ann D. Brow
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Electronic Patent Application Fee Transmittal

Application Number:	15634607
Filing Date:	27-Jun-2017
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Filer:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239/US-4/CON

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
LATE FILING FEE FOR OATH OR DECLARATION	1051	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**140**

Electronic Acknowledgement Receipt

EFS ID:	30653667
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow
Filer Authorized By:	
Attorney Docket Number:	EXCTD-35239/US-4/CON
Receipt Date:	13-OCT-2017
Filing Date:	27-JUN-2017
Time Stamp:	21:51:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$140
RAM confirmation Number	101617INTEFSW21532000
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		35239US4CON_SubSpecMarkup.pdf	501391 cdb3053fd1d7074550932b0bf0c61558e7a71840	yes	56

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Specification	1	55
Abstract	56	56

Warnings:

Information:

2		35239US4CON_SubSpecCleanb.pdf	498465 344c4f3dd691a75eb8440aaa4ac9663250c5a54f	yes	56

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Specification	1	55
Abstract	56	56

Warnings:

Information:

3		35239US4CON_RMP_10-13-17b.pdf	111556 845f6d8954e81d48386ee842990c51a6b4c82fe2	yes	7

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Applicant Response to Pre-Exam Formalities Notice	1	1
Specification	2	2

	Claims		3	5
	Applicant Arguments/Remarks Made in an Amendment		6	7
Warnings:				
Information:				
4	Fee Worksheet (SB06)	fee-info.pdf	30152 1bd0450d68c03d00330b6d5cf575a83f02a87993	no 2
Warnings:				
Information:				
		Total Files Size (in bytes):	1141564	

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 a 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.

~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

CROSS-REFERENCE TO RELATED APPLICATIONS

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

FIELD OF THE INVENTION

The present invention ~~is concerned with the diagnosis, staging and treatment of disease, in particular cancer and more specifically colorectal cancer. The invention~~ relates to methods and kits for processing and analysis of fecal samples comprising removing a portion of a collected fecal sample and adding the removed portion of the fecal sample to a buffer that prevents denaturation or degradation of blood proteins found in the sample, and comprising ~~diagnosing colorectal cancer based upon detecting epigenetic modifications, typically in specific genes. The methods and kits may also permit the~~ detection of blood in ~~a~~the removed portion of the fecal sample, *e.g.*, by immunoassay~~with the combined tests proving particularly advantageous~~.

BACKGROUND OF THE INVENTION

Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide, and is 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 perioxidase 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 protpoporphyrin 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 feacal 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 feacal 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.

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.

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.

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.

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

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.

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.

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).

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.

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,

(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.

a method for predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethyiating 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 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.

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.

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

(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.

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:

(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.

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

(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.

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 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; 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 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.

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 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 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 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 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 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 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 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 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 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. 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 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 (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.

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.

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.

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 metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase).

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.

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.

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 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 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 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 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 ~~fojlow~~ 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~~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 ~~a~~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 diferences 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 un methylated ~~cytosine~~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, SJ. (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 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 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 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 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 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. 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.

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 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®, MOLECULAR BEACONS®, AMPLIFLUOR® and SCORPION® DzyNA®, PlexorTM 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.

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 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.

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 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 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 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 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 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 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 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 ~~decribed~~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 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 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 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 ~~mimimal~~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.

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 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 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 (FOXE1), and 7 and 8 (NDRG4). In further 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 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 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 (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).

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 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. 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, 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 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, 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.

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.

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 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 ,

(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.

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 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.

~~Furthemore~~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 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.

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 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 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 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 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, 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 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-fluouro-2'-deoxycytidine, pseudoisocytidine, 5,6-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 CDOl 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 ~~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 (FOXE1), and 9 (NDRG4).

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 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 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 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 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 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. 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/~~purificatton~~purification of DNA. 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 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 to be detected as an indication of the ~~methtylation~~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:

(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 (FOXE1), 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 (FOXE1), 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 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 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 ~~methtylation~~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.

EXPERIMENTAL SECTION

Example 1

Sample collection and processing

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 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.

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 5 minutes at 2540 rcf at 20~~C~~°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 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 matrix. The supernatant was transferred into a new microcentrifuge tube. 25 µl of proteinase K was added to 60OuI 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 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 µl proteinase K to 2 ml of the supernatant, mix well, add 2.4 ml of AL Buffer and vortex for 15 s. Incubate at ~~7O0C~~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 µl 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 200µl 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~~0C~~°C for 15 minutes shaking at 1100 rpm. Then 100 µl of the diluted CT Conversion Reagent was added and samples were incubated at ~~7O0C~~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-HCI 1mM pH8.0 and stored at -80~~0C~~°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 5OuI) containing buffer (16.6mM (NH4)2SO4, 67 mM Tris (pH 8.8), 6.7 mM MgCI2, 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' - TTATTTTGCGAGCGGTTTC - 3	1
	reverse primer	5' - GAATACTCTAATTCCACGCGACT - 3'	2
	beacon	5'-FAM- CGACATGCGGGTTCGGTCGGCGCGGGGCATGTCG-3'-DABCYL	3
FOXE1	forward primer	5' - TTTGTTCGTTTTTCGATTGTTC - 3'	4
	reverse primer	5' - TAACGCTATAAAACTCCTACCGC - 3'	5
	beacon	5'-FAM- CGTCTCGTCGGGGTTCGGGCGTATTTTTTTAGGTAGGCGAGACG -3'-DABCYL	6
NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'	7
	reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 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-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-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'-GTTGGGTTTTCGTAGTTTTGTAGATCGC-3'	16
	Reverse primer	5'-CTACGCCCAAACTCGACG-3'	17
	beacon	5'-FAM-CGACATGCCCCGCCCTATCGCCGAAATCGCATGTCG-DABCYL-3'	18
SFRP2	Forward primer	5' – GGGTCGGAGTTTTTCGGAGTTGCGC – 3'	19
	Reverse primer	5' – CCGCTCTCTTCGCTAAATACGACTCG – 3'	20
	beacon	5'-FAM- CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTCGGGCATGTCG - 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-CGACATGCGTTCGTGTTTTGGTTTGTCGCGGTTTGGCATGTCG DABCYL-3'	27
LAMA1	Forward primer	5'-TTTTTAGATTTATCGAGTGGCG-3'	28
	Reverse primer	5'-CGAACTCACCTCTCTACCGAC-3'	29
	beacon	5'-CGACATGCCAAAAACACGCCCCCGCGCATGTCG-3'	30

MMP2	Forward primer	5'-TTCGGGTTATTAGCGTTTTTATC-3'	31
	Reverse primer	5'-ACTCCAACCAAACGACGAA-3'	32
	beacon	5'-FAM-CGACATCGTTGGTTCGGTGCGTGTGGTCGATGTCG-DABCYL-3'	33
CDO1	Forward primer	5'-AATTTGATTTGTGTGTGTATCGC-3'	34
	Reverse primer	5'-GAAACGTAAAAATATCGTCGCA-3'	35
	beacon	5'-FAM-CGACATGCGCGATTTCGGATTTATTGCGTTGTTAGGGCATGTCG-DABCYL-3'	36
TFPI2	Forward primer	5'-GTTCGTTGGGTAAGGCGTTC-3'	37
	Reverse primer	5'-CATAAAACGAACACCCGAACCG-3'	38
	Beacon	5'-FAM-CGACATGCACCGCGCACCTCCTCCCGCCAAGCATGTCG-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. 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%.

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.

		Methylation marker only		Methylation markers plus FIT (100 ng/ml cutoff)	
Gene	cutoffs (copies)	Spec (n=21)	Sens (n=23)	Spec (n=21)	Sens (n=23)
GATA4	10	90%	52%	86%	87%
GPNMB	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)	96%	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%
Sensivity for advanced adenoma	0%	0%	10%	10%	20%	10%	10%
Sensivity 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%
Sensivity for advanced adenoma	10%
Sensivity 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~~0C~~°C until further use.

In this study only a portion of the stool sample ~~(« 1g)~~ (≈ 1g) was investigated for further methylation testing. First, historical samples were thawed at the storage site and partial samples were taken ~~(= "Ig)~~ (≈ 1g) using special designed stool tubes with integrated measuring spoons (Sarstedt). Each sample was then weighted and 2 volumes of stabilizing buffer (Diagn MoI Pathol; Volume 14, Number 3, September 2005) were added. Samples were stored at -80~~0C~~°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 250µl of homogenized stool material each, and elute in 75µl of buffer 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

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 2µg of input DNA was used and elution was done in 30µl Tris-HCI (1mM, pH 8.0); samples were stored at ~~-8O0C~~-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 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.

ABSTRACT

A method of ~~detecting a predisposition to, or the incidence of, colorectal cancer in~~processing a fecal sample from a human subject~~comprises, in a first step (a),~~ 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~~, wherein detection of the presence of blood is indicative of a predisposition to, or the incidence of, colorectal cancer. The method additionally comprises, in second step (b), detecting an epigenetic modification in the DNA contained within the fecal sample, wherein detection of the epigenetic modification is indicative of a predisposition to, or the incidence of, colorectal cancer. Based upon a positive result obtained in either (a) or (b) or in both (a) and (b) a predisposition to, or the incidence of, colorectal cancer is detected. Related methods and kits involve detecting an epigenetic modification in a number of specific genes~~. The method further comprises stabilizing the remaining portion of the fecal sample.

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

CROSS-REFERENCE TO RELATED APPLICATIONS

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

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 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 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 perioxidase 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 protpoporphyrin 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 feacal 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 feacal 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.

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.

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.

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.

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

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.

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.

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).

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.

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,

(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.

a method for predicting the likelihood of resistance to treatment of colorectal cancer with a DNA demethyiating 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 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.

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.

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

(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.

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:

(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.

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

(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.

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 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; 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 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.

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 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 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 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 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 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 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 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 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 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. 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 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 (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.

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.

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.

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 metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase).

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.

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.

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 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 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 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 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 diferences 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 un methylated 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, SJ. (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 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 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 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 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 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. 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.

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 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®, MOLECULAR BEACONS®, AMPLIFLUOR® and SCORPION® DzyNA®, PlexorTM 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.

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 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.

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 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 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 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 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 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 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 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 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 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 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.

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 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 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 (FOXE1), and 7 and 8 (NDRG4). In further 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 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 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 (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).

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 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. 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, 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 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, 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.

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.

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 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 ,

(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.

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 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 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.

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 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 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 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 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, 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 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-fluouro-2'-deoxycytidine, pseudoisocytidine, 5,6-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 CDOl 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 ~~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 (FOXE1), and 9 (NDRG4).

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 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 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 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 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 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. 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 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 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:

(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 (FOXE1), 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 (FOXE1), 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 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 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. More specifically, the bisulphite reagent may comprise, consist essentially of or consist of sodium bisulphite.

EXPERIMENTAL SECTION

Example 1

Sample collection and processing

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 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.

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 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 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 matrix. The supernatant was transferred into a new microcentrifuge tube. 25 µl of proteinase K was added to 60OuI 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 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 µl 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 µl 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 200µl 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-HCI 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 5OuI) containing buffer (16.6mM (NH4)2SO4, 67 mM Tris (pH 8.8), 6.7 mM MgCI2, 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' - TTATTTTGCGAGCGGTTTC - 3	1
	reverse primer	5' - GAATACTCTAATTCCACGCGACT - 3'	2
	beacon	5'-FAM- CGACATGCGGGTTCGGTCGGCGCGGGGCATGTCG-3'-DABCYL	3
FOXE1	forward primer	5' - TTTGTTCGTTTTTCGATTGTTC - 3'	4
	reverse primer	5' - TAACGCTATAAAACTCCTACCGC - 3'	5
	beacon	5'-FAM- CGTCTCGTCGGGGTTCGGGCGTATTTTTTTAGGTAGGCGAGACG -3'-DABCYL	6
NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'	7
	reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 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-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-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'-GTTGGGTTTTCGTAGTTTTGTAGATCGC-3'	16
	Reverse primer	5'-CTACGCCCAAACTCGACG-3'	17
	beacon	5'-FAM-CGACATGCCCCGCCCTATCGCCGAAATCGCATGTCG-DABCYL-3'	18
SFRP2	Forward primer	5' – GGGTCGGAGTTTTTCGGAGTTGCGC – 3'	19
	Reverse primer	5' – CCGCTCTCTTCGCTAAATACGACTCG – 3'	20
	beacon	5'-FAM- CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTCGGGCATGTCG - 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-CGACATGCGTTCGTGTTTTGGTTTGTCGCGGTTTGGCATGTCG DABCYL-3'	27
LAMA1	Forward primer	5'-TTTTTAGATTTATCGAGTGGCG-3'	28
	Reverse primer	5'-CGAACTCACCTCTCTACCGAC-3'	29
	beacon	5'-CGACATGCCAAAAACACGCCCCCGCGCATGTCG-3'	30

MMP2	Forward primer	5'-TTCGGGTTATTAGCGTTTTTATC-3'	31
	Reverse primer	5'-ACTCCAACCAAACGACGAA-3'	32
	beacon	5'-FAM-CGACATCGTTGGTTCGGTGCGTGTGGTCGATGTCG-DABCYL-3'	33
CDO1	Forward primer	5'-AATTTGATTTGTGTGTGTATCGC-3'	34
	Reverse primer	5'-GAAACGTAAAAATATCGTCGCA-3'	35
	beacon	5'-FAM-CGACATGCGCGATTTCGGATTTATTGCGTTGTTAGGGCATGTCG-DABCYL-3'	36
TFPI2	Forward primer	5'-GTTCGTTGGGTAAGGCGTTC-3'	37
	Reverse primer	5'-CATAAAACGAACACCCGAACCG-3'	38
	Beacon	5'-FAM-CGACATGCACCGCGCACCTCCTCCCGCCAAGCATGTCG-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. 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%.

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.

		Methylation marker only		Methylation markers plus FIT (100 ng/ml cutoff)	
Gene	cutoffs (copies)	Spec (n=21)	Sens (n=23)	Spec (n=21)	Sens (n=23)
GATA4	10	90%	52%	86%	87%
GPNMB	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)	96%	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%
Sensivity for advanced adenoma	0%	0%	10%	10%	20%	10%	10%
Sensivity 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%
Sensivity for advanced adenoma	10%
Sensivity 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.

In this study only a portion of the stool sample (≈ 1g) was investigated for further methylation testing. First, historical samples were thawed at the storage site and partial samples were taken (≈ 1g) using special designed stool tubes with integrated measuring spoons (Sarstedt). Each sample was then weighted and 2 volumes of stabilizing buffer (Diagn 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 250µl of homogenized stool material each, and elute in 75µl of buffer 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

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 2µg of input DNA was used and elution was done in 30µl Tris-HCI (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 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.

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.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/634,607

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	19 minus 20 =	*			OR	x 80 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*				x 420 =	0.00
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).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1600

APPLICATION AS AMENDED - PART II

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

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
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	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
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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
15/634,607	06/27/2017	1634	1740	EXCTD-35239/US-4/CON	19	2

CONFIRMATION NO. 1874

UPDATED FILING RECEIPT

72960
Casimir Jones, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

OC000000094769682

Date Mailed: 10/17/2017

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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 15/010,436 01/29/2016
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.
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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/634,607**

Projected Publication Date: 10/19/2017

Non-Publication Request: No

Early Publication Request: No

Title

METHODS FOR DETECTING COLORECTAL CANCER

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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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239/US-4/CON

CONFIRMATION NO. 1874

72960
Casimir Jones, S.C.
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MIDDLETON, WI 53562

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239/US-4/CON

CONFIRMATION NO. 1874

72960
Casimir Jones, S.C.
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PUBLICATION NOTICE



Title:METHODS FOR DETECTING COLORECTAL CANCER

Publication No.US-2017-0298448-A1
Publication Date:10/19/2017

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Attorney Docket Number: EXCTD-35239/US-4/CON

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In re Application of:	JOOST, Louwagie	Confirmation No.:	1874
Serial No.:	15/634,607	Group No.:	1634
Filed:	27-Jun-2017	Examiner:	N/A
Entitled:	**METHODS OF DETECTING COLORECTAL CANCER**		

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Respectfully submitted,

Date: October 24, 2017

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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 15/010,436 filed on 29-Jan-2016.

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: Joost Louwagie Date (Optional): 23 Jan 2016

Signature: [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/SB/AIA01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. 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 1 minute 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.**

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

Electronic Acknowledgement Receipt

EFS ID:	30748980
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Lisa M. Day
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239/US-4/CON
Receipt Date:	24-OCT-2017
Filing Date:	27-JUN-2017
Time Stamp:	18:13:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	35239US4CON_DEC_Transmittal.pdf	78561 04ba338312ea22053cf1f1189280a58bb5dfa057	no	1

Warnings:

Information:					
2	Oath or Declaration filed	35239US4CON_Exec_Decl.pdf	108303 3b075e2783286710203e5fae24e5b07b59f5995e	no	1

Warnings:	
Information:	
Total Files Size (in bytes):	186864

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 a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

Patent Owner's Ex. 2003, Page 241

CORRECTED ADS FORM

Application Number	15634607
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER

Inventor Information

****If no data is shown, no data has been corrected****

	Data of Record	Updated Data
Order Number		
Name		
Residence Information		
Residency		
City		
State		
Country of Residence		
Mailing Address of Inventor		
Address 1		
Address 2		
City,State/Province, Postal Code		
Country		

Patent Owner's Ex. 2003, Page 242

Application Information

	Data of Record	Updated Data
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	
Attorney Docket Number	EXCTD-35239/US-4/CON	
Entity Type	Regular Undiscounted	

Domestic Benefit/National Stage Information

****If no data is shown, no data has been corrected****

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121,365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing this 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).

		Data of Record	Updated Data
	Prior Application Status		
	Application Number		
	Continuity Type		
	Prior Application Number		
	Filing Date (YYYY-MM-DD)		
	Patent Number		
	Issue Date (YYYY-MM-DD)		

Foreign Priority Information

Patent Owner's Ex. 2003, Page 243

****If no data is shown, no data has been corrected****

This section allows for the applicant to claim priority to a foreign application. 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(i)(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).

		Data of Record	Updated Data
	Application Number		
	Country		
	Filing Date		
	Access Code		

Applicant Information

****If no data is shown, no data has been corrected****
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 of Record	Updated Data
Applicant Type	ASG	
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is		
Name of the Deceased or Legally Incapacitated Inventor		
Applicant is an Organization	Yes	
Name		
Organization Name	~~Exact Sciences Corporation~~	EXACT SCIENCES DEVELOPMENT COMPANY, LLC
Address 1		441 Charmany Drive
Address 2		

Patent Owner's Ex. 2003, Page 244

City,State/Province,Postal Code	~~Madison WI~~	Madison WI 53719
Country	US	
Phone Number		
Fax Number		
Email Address		

Assignee Information including Non-Applicant Assignee Information

****If no data is shown, no data has been corrected****
Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office

	Data of Record	Updated Data
Order		
Applicant is an Organization		
Name		
Organization Name		
Mailing Address		
Address 1		
Address 2		
City,State/Province,Postal Code		
Country		
Phone Number		
Fax Number		
Email Address		

Patent Owner's Ex. 2003, Page 245

Signature

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b).

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

Signature	/Mary Ann D. Brow/	Registration Number	42363
First Name	Mary Ann D.	Last Name	Brow

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
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.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Exact Sciences Development Company, LLC

Application No./Patent No.: 15/335,111 Filed/Issue Date: 10/26/2016

Titled: ISOLATION AND DETECTION OF DNA FROM PLASMA

Exact Sciences Development Company, LLC, a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. ☑ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Hatim Allawi et al. To: Exact Sciences Corporation

   The document was recorded in the United States Patent and Trademark Office at
   Reel 040872, Frame 0369, or for which a copy thereof is attached.

2. From: Exact Sciences Corporation To: Exact Sciences Development Company, LLC

   The document was recorded in the United States Patent and Trademark Office at
   Reel 044119, Frame 0001, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
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.

STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Mary Ann D. Brow/	2018-07-23
Signature	Date
Mary Ann D. Brow	42363
Printed or Typed Name	Title or Registration Number

Privacy Act Statement

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

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

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

Doc Code: R46C.REQ

Document Description: Request under 37 CFR 1.46(c) to correct, update or change the applicant.

PTO/AIA/41 (04-15)
Approved for use through 11/30/2020. OMB 0651-0031
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.

REQUEST TO CORRECT OR UPDATE THE NAME OF THE APPLICANT UNDER 37 CFR 1.46(c)(1), OR CHANGE THE APPLICANT UNDER 37 CFR 1.46(c)(2)

(FOR USE ONLY IN APPLICATIONS FILED ON OR AFTER SEPTEMBER 16, 2012)

Application Number	15/335,111
Filing Date	10/26/2016
First Named Inventor	Allawi
Art Unit	
Examiner Name	
Practitioner Docket Number	EXCTD-35068/US-1/ORD

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Applicant hereby **requests that the name of the applicant be corrected or updated under 37 CFR 1.46(c)(1), or that the applicant be changed under 37 CFR 1.46(c)(2),** in the above-identified application. **Requests under 37 CFR 1.46(c)(1) or (c)(2) cannot be submitted after payment of the issue fee or if the application has been patented.**

Please check the applicable box(es) below.

☐ 1. This request is to **correct or update the name of the applicant** (**under 37 CFR 1.46(c)(1)**) and includes:

☐ An application data sheet (ADS) in accordance with 37 CFR 1.76(c) with the corrected or updated information shown with markings (*e.g.*, underlining for insertions, strikethrough for deletions). A Corrected Web-based ADS may be used.

Note: Requests under 37 CFR 1.46(c)(1) may be filed to correct typographical errors in the name of the § 1.46 applicant, or for updating the name of the § 1.46 applicant (*i.e.*, where there is no change in the applicant itself but just in the applicant's name). See the Manual of Patent Examining Procedure (MPEP) section 605.01.

■ 2. This request is to **change the applicant** (**under 37 CFR 1.46(c)(2)**) and includes:

■ An application data sheet (ADS) in accordance with 37 CFR 1.76(c) that identifies the changes with proper markings (underlining for insertions and strikethrough for deletions). A Corrected Web-based ADS may be used.

■ A Statement Under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent). See MPEP 325.

I am the

☐ applicant* ■ attorney or agent of record Registration number 42363 ☐ attorney or agent acting under 37 CFR 1.34 Registration number _____

Signature /Mary Ann D. Brow/

Typed or printed name Mary Ann D. Brow

Date 07/232018

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. *Juristic entities must be represented by a patent practitioner (See 37 CFR 1.31, applicable to any paper filed on or after September 16, 2012 that is presented on behalf of a juristic entity, regardless of application filing date). Submit multiple forms if more than one signature is required, see below**.

■ ** Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.36. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

Privacy Act Statement

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

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

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

Electronic Acknowledgement Receipt

EFS ID:	33252620
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239/US-4/CON
Receipt Date:	23-JUL-2018
Filing Date:	27-JUN-2017
Time Stamp:	14:17:40
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet to update/ correct info	CorrectedADS.pdf	65284 2177168e3d3c2cecb9c1503bd0c30868593af7df	no	5

Warnings:

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	2018-07-23_34810US2ORD_Exact_373.pdf	121444 c9ac1efa5375162b3f2397a9d748b5f77f9cd1cf	no	3
Warnings:					
Information:					
3	Request for Corrected Filing Receipt	2018-07-23_35068US1ORD_Applicant_Change.pdf	213289 0b1dba8ecfc71d332089ac22547d6a8852b08e89	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	400017		

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 a 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	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/634,607	06/27/2017	1634	1740	EXCTD-35239/US-4/CON	19	2

CONFIRMATION NO. 1874

CORRECTED FILING RECEIPT

72960
Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562

OC000000101213749

Date Mailed: 07/26/2018

Receipt is acknowledged of this non-provisional 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 APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Joost Louwagie, Dornach, SWITZERLAND;

Applicant(s)

EXACT SCIENCES DEVELOPMENT COMPANY, LLC, Madison, WI;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application 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: 07/12/2017

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/634,607**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

Title

METHODS FOR DETECTING COLORECTAL CANCER

Preliminary Class

435

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).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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 AssetsControl, 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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PTO/AIA/80 (07-17)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

[X] Practitioners associated with Customer Number: 72960

OR

[ ] Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

[X] The address associated with Customer Number:

OR

[ ]

Firm or individual name		
Address		
City	State	Zip
Country		
Telephone	Email	

Assignee name and address: EXACT SCIENCES DEVELOPMENT COMPANY, LLC

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee.

Signature Gretchen E. Cleland	Date Jan. 11, 2018
Name Gretchen E. Cleland	Telephone
Title Associate General Counsel	

This collection of information is required by 37 CFR 1.31, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public, which is to update (and by the USPTO to process) the file of a patent or reexamination proceeding. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 18 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.**

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

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
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.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Exact Sciences Development Company, LLC

Application No./Patent No.: 15/634,607 Filed/Issue Date: 06/27/2017

Titled: METHODS FOR DETECTING COLORECTAL CANCER

Exact Sciences Development Company, LLC, a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. ☑ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Louwagie Joost To: MDxHealth SA

   The document was recorded in the United States Patent and Trademark Office at

   Reel 027951, Frame 0162, or for which a copy thereof is attached.

2. From: MDxHealth SA To: EXACT SCIENCES CORPORATION

   The document was recorded in the United States Patent and Trademark Office at

   Reel 042136, Frame 0519, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
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.

STATEMENT UNDER 37 CFR 3.73(c)

3. From: EXACT SCIENCES CORPORATION To: EXACT SCIENCES DEVELOPMENT COMPANY, LLC

The document was recorded in the United States Patent and Trademark Office at
Reel 044119, Frame 0001, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Mary Ann D. Brow/	2018-07-23
Signature	Date
Mary Ann D. Brow	42363
Printed or Typed Name	Title or Registration Number

Privacy Act Statement

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

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

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

Electronic Acknowledgement Receipt

EFS ID:	33289209
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239/US-4/CON
Receipt Date:	26-JUL-2018
Filing Date:	27-JUN-2017
Time Stamp:	11:41:56
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	EXCTD_EXEC_GEN_POA_80.pdf	424277 4011c970f6654ae46ec092ca6276e9dd2d0ce41b	no	1

Warnings:

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	2018-07-23_35239US4CON_373.pdf	122253 6b6931a0da09a204c7bfb34fbfe9fd85f1510b6c	no	3
Warnings:					
Information:					
			Total Files Size (in bytes):	546530	

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 a 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.

To: docketing@casimirjones.com,pto.correspondence@casimirjones.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 72960

Jul 26, 2018 04:06:50 AM

Dear PAIR Customer:

Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 72960 , 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.
15634607	APP.FILE.REC	07/26/2018	EXCTD-35239/US-4/CON

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

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
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239/US-4/CON

CONFIRMATION NO. 1874

72960
Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562

POA ACCEPTANCE LETTER

OC000000101363971

Date Mailed: 08/01/2018

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 07/26/2018.

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**.

/nbekele/

To: docketing@casimirjones.com,pto.correspondence@casimirjones.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 72960

Aug 01, 2018 03:42:24 AM

Dear PAIR Customer:

Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 72960 , 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.
15634607	N570	08/01/2018	EXCTD-35239/US-4/CON

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239/US-4/CON	1874

72960 7590 03/22/2019

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

EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
03/22/2019	ELECTRONIC

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

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

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

docketing@casimirjones.com
pto.correspondence@casimirjones.com

Office Action Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Bradley L Sisson	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 2 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 10/13/2017.

☐ 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) 82-100 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) _____ is/are rejected.

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

9) ☑ Claim(s) 82-100 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)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Election/Restrictions

2. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 82-84 and 87-100, drawn to a method of processing a fecal sample and analyzing DNA extracted, classified in class 436, subclass 94.

II. Claims 85 and 86, drawn to a set of articles for collection of a fecal sample at home by a human subject, classified in class 206, subclass 581.

3. The inventions are independent or distinct, each from the other because:

Inventions II and I are directed to an unrelated product and process. Product and process inventions are unrelated if it can be shown that the product cannot be used in, or made by, the process. See MPEP § 802.01 and § 806.06. In the instant case, the method of processing the fecal sample, including the DNA analysis, does not require the set of articles specified in Group II, claims 85 and 86.

4. Restriction for examination purposes as indicated is proper because all the inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and/or examination burden if restriction were not required because one or more of the following reasons apply:

- the inventions have acquired a separate status in the art in view of their different classification
- the inventions have acquired a separate status in the art due to their recognized divergent subject matter

- the inventions require a different field of search (e.g., searching different classes /subclasses or electronic resources, or employing different search strategies or search queries).

5. **Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined** even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

6. The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

7. Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other invention.

Species election in Group I

8. In the event that applicant elects the invention of Group I, the following election of species will apply.

9. This application contains claim 99, which is directed to the following patentably distinct species of genes: PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2, and CDO1. The species are independent or distinct because they encode different proteins, have different functions, and are comprised of different nucleotide sequences. In addition, these species are not obvious variants of each other based on the current record.

10. Applicant is required under 35 U.S.C. 121 to elect a single disclosed species, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 87, 95, and 98 are generic.

11. There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply:

- the inventions have acquired a separate status in the art due to their recognized divergent subject matter
- the inventions require a different field of search (e.g., searching different classes /subclasses or electronic resources, or employing different search strategies or search queries).

12. **Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined** even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species or grouping of patentably indistinct species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

13. The election may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out

supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

14. Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing them to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other species.

15. Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

Conclusion

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Bradley L. Sisson whose telephone number is (571)272-0751. The examiner can normally be reached on Monday to Thursday, from 6:30 AM to 5 PM..

17. Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

18. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on 571-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

19. 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.

/Bradley L. Sisson/
Primary Examiner, Art Unit 1634

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	Art Unit
	Bradley L Sisson	1634

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

CLAIMS

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

CLAIM		DATE								
Final	Original	03/18/2019								
	1	-								
	2	-								
	3	-								
	4	-								
	5	-								
	6	-								
	7	-								
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

CLAIM		DATE								
Final	Original	03/18/2019								
	43	-								
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

CLAIM		DATE								
Final	Original	03/18/2019								
	95	÷								
	96	÷								
	97	÷								
	98	÷								
	99	÷								
	100	÷								

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

BIB DATA SHEET

CONFIRMATION NO. 1874

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/634,607	06/27/2017 RULE	435	1634	EXCTD-35239/US-4/CON

APPLICANTS

EXACT SCIENCES DEVELOPMENT COMPANY, LLC, Madison, WI;

INVENTORS

Joost Louwagie, Dornach, SWITZERLAND;

**** CONTINUING DATA ***************************

This application 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 ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

07/12/2017

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
❑ Yes ☑ No	❑ Yes ☑ No	/BRADLEY L SISSON/ Examiner's Signature	❑ Initials	SWITZERLAND	0	19	2

ADDRESS

Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562
UNITED STATES

TITLE

METHODS FOR DETECTING COLORECTAL CANCER

FILING FEE RECEIVED		
1740	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other _____ ❑ Credit

To: docketing@casimirjones.com,pto.correspondence@casimirjones.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 72960

Mar 22, 2019 04:01:22 AM

Dear PAIR Customer:

Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 72960 , 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.
15634607	CTRS	03/22/2019	EXCTD-35239/US-4/CON

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

PATENT
Attorney Docket No.: **EXCTD-35239/US-4/CON**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	1874
Serial No.:	15/634,607	Group No.:	1634
Filed:	06/27/2017	Examiner:	Bradley L. Sisson
Entitled:	**METHODS FOR DETECTING COLORECTAL CANCER**		

AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT MAILED 03/22/2019

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

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

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

Dated: May 22, 2019 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This paper is responsive to the Restriction Requirement mailed 03/22/2019, with response due by 05/22/2019.

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/US-4/CON**. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

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

Remarks begin on Page 5.

AMENDMENTS TO THE CLAIMS:

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

1-81. (cancelled)

82. (previously presented) A method of processing a fecal sample, the method comprising

a) collecting a fecal sample from a human subject;

b) removing a portion of said fecal sample to produce a removed portion and a remaining portion of said fecal sample;

c) combining the removed portion with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample;

d) stabilizing the remaining portion of the fecal sample.

83. (previously presented) The method of claim 82, wherein said stabilizing of said remaining portion of the fecal sample comprises one or both of:

i) adding a stabilizing buffer to said remaining sample;

ii) freezing said remaining sample.

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

85-86. (cancelled)

87. (previously presented) A method of processing a fecal sample, the method comprising:

A) obtaining a pair of portions of a fecal sample from a human subject, the pair of portions comprising a removed portion and a stabilized remaining portion of a fecal sample obtained by the method of claim 82, and

B) testing the removed portion of said fecal sample for the presence of blood.

88. (previously presented) The method of claim 87, wherein said testing comprises testing

the removed portion of said fecal sample for a concentration of hemoglobin present in said removed portion.

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

90. (previously presented) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml.

91. (previously presented) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml.

92. (previously presented) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml.

93. (previously presented) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 50 ng/ml.

94. (previously presented) The method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml.

95. (previously presented) The method of claim 87, further comprising

C) extracting DNA from the stabilized remaining portion of said fecal sample; and

D) analyzing said DNA for the presence of DNA from a human, wherein the

DNA from a human has an epigenetic modification.

96. (previously presented) The method of claim 95, wherein said epigenetic modification comprises aberrant methylation.

97. (previously presented) The method of claim 95, wherein said aberrant methylation comprises hypermethylation.

98. (previously presented) The method of claim 95, wherein said DNA from a human comprises a gene and/or a promoter region of a gene.

99. (previously presented) The method of claim 98, wherein said gene selected from the group consisting of PHACTR3, NDRG4, FOXE1 , GATA4, GPNMB, TFPI2, SOX17, SYNE1 , LAMA, MMP2, OSMR, SFRP2, and CDO1.

100. (previously presented) The method of claim 96, wherein analyzing said DNA comprises modifying said DNA with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.

REMARKS

Claims 88-100 are pending in the Application. In the Restriction Requirement mailed 03/22/2019, the Examiner restricted the claims into the following two groups:

I. Claims 82-84 and 87-100, drawn to a method of processing a fecal sample and analyzing DNA extracted; and

II. Claims 85 and 86, drawn to a set of articles for collection of a fecal sample at home by a human subject.

For business reasons and without acquiescing to the Examiner's arguments, Applicant herein elects the claims of Group I, without traverse. Claims 85 and 86 are cancelled in view of the election, reserving the right to file the same or similar claims in one or more divisional applications.

The Examiner has further required an election of species of one specific gene from claim 99: PHACTR3, NDRG4, FOXE1 , GATA4, GPNMB, TFPI2, SOX17, SYNE1 , LAMA, MMP2, OSMR, SFRP2, and CDO1. Applicant hereby elects NDRG4, reading on claims 82-84 and 87-100, with claims 82, 87, and 95 being generic. Applicant reserves all rights to rejoinder and to examination of additional species upon a finding of allowability of a generic claim.

If the Examiner wishes to discuss this case, Applicant encourages the Examiner to call the undersigned at 608-662-1277 at the Examiner's convenience.

Dated: May 22, 2019

/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

EFS ID:	36090889
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow
Filer Authorized By:	
Attorney Docket Number:	EXCTD-35239/US-4/CON
Receipt Date:	22-MAY-2019
Filing Date:	27-JUN-2017
Time Stamp:	18:49:22
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2019-05-22_35239US4CON_RRR.pdf	107669 48cd3db89445b7a00518760304a9aaeb55f2b67c	yes	5

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Response to Election / Restriction Filed	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	5

Warnings:	
Information:	
Total Files Size (in bytes):	107669

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 a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PATENT
Attorney Docket No.: **EXCTD-35239/US-4/CON**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

SUPPLEMENTAL AMENDMENT

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

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

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

Dated: May 23, 2019 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This paper is a Supplemental Amendment to the Amendment and Response to filed 05/22/2019.

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/US-4/CON**. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Amendment to the Specification is on on Page 2;

Remarks are on Page 3.

AMENDMENT TO THE SPECIFICATION

On page 1 of the specification, please replace the title at line 1 with the following amended title:

~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

REMARKS

The specification is amended herein to provide a descriptive title of the claimed subject matter. No new matter is added. A corrected Application Data Sheet is filed herewith.

If the Examiner wishes to discuss this case, Applicant encourages the Examiner to call the undersigned at 608-662-1277 at the Examiner's convenience.

Dated: May 23, 2019 /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

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. 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 contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	

Title of Invention: ~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

The application data sheet is part of the provisional or nonprovisional 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.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Joost		Louwagie	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Dornach	Country of Residence	CH

Mailing Address of Inventor:

Address 1	441 Charmany Drive		
Address 2			
City	Madison	**State/Province**	WI
Postal Code	53719	**Country**	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	72960		
Email Address	docketing@casimirjones.com	Add Email	Remove Email

Application Information: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Title of the Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~		
Attorney Docket Number	EXCT-35239/US-4/CON	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)		**Suggested Figure for Publication (if any)**	

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. 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 contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

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).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	72960		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 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.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	15010436	2016-01-29

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
15010436	Continuation of	13147570	2012-03-12
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13147570	a 371 of international	PCT/GB2010/000180	2010-02-03
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/GB2010/000180	Claims benefit of provisional	61149581	2009-02-03

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. 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)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(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).

			Remove
Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ 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.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
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Title of Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed 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)

A. 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).

B. 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).

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 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.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

Applicant Information:

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.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. 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. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Exact Sciences Development Company, LLC		
Mailing Address Information For Applicant:			
Address 1	441 Charmany Drive		
Address 2			
City	Madison	**State/Province**	WI
Country	US	Postal Code	53719
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

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.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

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

Signature	/Mary Ann D. Brow/			Date (YYYY-MM-DD)	2019-05-23
First Name	Mary Ann D.	Last Name	Brow	Registration Number	42363

Additional Signature may be generated within this form by selecting the Add button.

Patent Owner's Ex. 2003, Page 293
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	~~METHODS FOR DETECTING COLORECTAL CANCER~~ FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

This collection of information is required by 37 CFR 1.76. 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 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet 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.**

Privacy Act Statement

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

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

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

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

EFS ID:	36102362
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	METHODS FOR DETECTING COLORECTAL CANCER
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239/US-4/CON
Receipt Date:	23-MAY-2019
Filing Date:	27-JUN-2017
Time Stamp:	21:25:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	2019-05-23_35239US4CON_IDS_Letter.pdf	95814 33e6c56e04409733b03b9f279a6404039ba9a60e	no	2

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	2019-05-23_35239US4CON_IDS-SB08.pdf	172667 030704dc6026247c5118d27dda77581d9c1ae761	no	6
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3		2019-05-23_35239US4CON_SuppleAmd.pdf	105432 566434b8f6d84c202adefe78cdc2050d282c4f04	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Supplemental Response or Supplemental Amendment	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:					
Information:					
4	Application Data Sheet	35239US4CON_Corrected_Title ADS-Markedup.pdf	126485 e7e15b6bffc06a955d3d52359a941227acd7f1a7	no	8
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
			Total Files Size (in bytes):	500398	

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 a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PATENT
Attorney Docket No.: EXCT-35239/US-4/CON

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

INFORMATION DISCLOSURE STATEMENT

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

Sir or Madam:

The citations listed in the attached IDS Form SB08A may be material to the examination of the above-identified application, and are therefore submitted in compliance with the duty of disclosure defined in 37 C.F.R. §§ 1.56 and 1.97.

Applicants wish to bring to the Examiner's attention that we are not providing copies of US Patents or published US patent applications as instructed under 37 CFR 1.98(a)(2). Copies of any cited foreign publications and non-patent literature are not provided herewith as they are available in the Image File Wrapper of parent U.S. Patent Application 13/147,570, filed March 12, 2012. The Examiner is requested to make these citations of official record in this application.

This Information Disclosure Statement under 37 C.F.R. §§ 1.56 and 1.97 is not to be construed as a representation that a search has been made, that additional information material to the examination of this application does not exist, or that any one or more of these citations constitutes prior art.

Dated: May 23, 2019

/Mary Ann D. Brow/
Mary Ann D. Brow
Registration No. 42363
Casimir Jones s.c.
2275 Deming Way, Suite 310
Middleton, WI 53562

(608) 662-1277

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	1	of	5	Attorney Docket Number	EXCTD-35239/US-4/CON

U.S. PATENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		4437975	1984-03-20	GILLESPIE et al.	
		4582811	1986-04-15	PUCCI et al.	
		4683197	1987-07-28	GALLATI	
		4889818	1989-12-26	GELFAND 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

Examiner Initials*	Cite No.[3]	Document Number Number-Kind Code[4] (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20020187476	2002-12-12	KOROULIS et al.	
		20040091881	2004-05-13	OLEK et al.	
		20080221056	2008-09-11	BAYLIN et al.	
		20080227208	2008-09-18	YEE 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 Country Code[5] Number[6] Kind Code[7] (if known)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translation[8]

Examiner Signature		Date Considered	

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 (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	2	of	5	Attorney Docket Number	EXCTD-35239/US-4/CON

FOREIGN PATENT DOCUMENTS						
		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 2004/0927209	2004-10-28	KIETURAKIS & KARCZEWSKI		
		WO 2005/017207	2005-02-04	CASE WESTERN RESERVE UNIVERSITY		
		WO 2006/113671	2006-10-26	ONCOMETHYLOME SCIENCES SA		
		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	

Examiner Signature		Date Considered	

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 (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	3	of	5	Attorney Docket Number	EXCTD-35239/US-4/CON

NONPATENT LITERATURE DOCUMENTS			
		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.	
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		SOMMER et al., Minimal homology requirements for PCR primers. Nucleic Acids Res. 1989 Aug 25;17(16):6749	

Examiner Signature		Date Considered	

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 (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	4	of	5	Attorney Docket Number	EXCTD-35239/US-4/CON

NONPATENT LITERATURE DOCUMENTS			
		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	

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 (Use as many sheets as necessary)				Complete if Known	
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				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	5	of	5	*Attorney Docket Number*	EXCTD-35239/US-4/CON

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.

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)	2019-05-23
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.

[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**.

PTO/SB/06 (09-11)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/634,607	Filing Date 06/27/2017	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ 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).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/CAROLYN E THOMAS/
*** 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 number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. 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 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. 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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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/634,607	Filing Date 06/27/2017	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ 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).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	05/22/2019	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 17	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/CORALIA BETANCOURT/
*** 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 number found in the appropriate box in column 1.	

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239/US-4/CON	1874

72960 7590 06/27/2019

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

EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
06/27/2019	ELECTRONIC

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

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

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

docketing@casimirjones.com
pto.correspondence@casimirjones.com

Office Action Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Bradley L Sisson	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 22 May 2019.

☐ 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) 82-84 and 87-100 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) 82-84 and 87-100 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)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 23 May 2019.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Claim Interpretation

2. Attention is directed to MPEP 904.01 [R-08.2012].

> The breadth of the claims in the application should always be carefully noted; that is, the examiner should be fully aware of what the claims do not call for, as well as what they do require. During patent examination, the claims are given the broadest reasonable interpretation consistent with the specification. See *In re Morris*, 127 F.3d 1048, 44 USPQ2d 1023 (Fed. Cir. 1997). See MPEP § 2111 - § 2116.01 for case law pertinent to claim analysis.

3. It is noted with particularity that narrowing limitations found in the specification cannot be inferred in the claims where the elements not set forth in the claims are linchpin of patentability. *In re Philips Industries v. State Stove & Mfg. Co, Inc.*, 186 USPQ 458 (CA6 1975). While the claims are to be interpreted in light of the specification, it does not follow that limitations from the specification may be read into the claims. On the contrary, claims must be interpreted as broadly as their terms reasonably allow. See *Ex parte Oetiker,* 23 USPQ2d 1641 (BPAI, 1992). In added support of this position, attention is directed to MPEP 2111 [R-11.2013], where, citing *In re Prater*, 415 F.2d 1393, 1404-05, 162 USPQ 541, 550-51 (CCPA 1969), is stated:

> The court explained that "reading a claim in light of the specification, to thereby interpret limitations explicitly recited in the claim, is a quite different thing from 'reading limitations of the specification into a claim,' to thereby narrow the scope of the claim by implicitly adding disclosed limitations which have no express basis in the claim." The

court found that applicant was advocating the latter, i.e., the impermissible importation of subject matter from the specification into the claim.

4. Additionally, attention is directed to MPEP 2111.01 [R-07.2015], wherein is stated:

II. IT IS IMPROPER TO IMPORT CLAIM LIMITATIONS FROM THE SPECIFICATION

"Though understanding the claim language may be aided by explanations contained in the written description, it is important not to import into a claim limitations that are not part of the claim. For example, a particular embodiment appearing in the written description may not be read into a claim when the claim language is broader than the embodiment." *Superguide Corp. v. DirecTV Enterprises, Inc.*, 358 F.3d 870, 875, 69 USPQ2d 1865, 1868 (Fed. Cir. 2004).

5. Attention is also directed to MPEP 2111.02 II. As stated herein:

II. PREAMBLE STATEMENTS RECITING PURPOSE OR INTENDED USE
The claim preamble must be read in the context of the entire claim. The determination of whether preamble recitations are structural limitations or mere statements of purpose or use "can be resolved only on review of the entirety of the [record] to gain an understanding of what the inventors actually invented and intended to encompass by the claim." *Corning Glass Works,* 868 F.2d at 1257, 9 USPQ2d at 1966. **If the body of a claim fully and intrinsically sets forth all of the limitations of the claimed invention, and the preamble merely states, for example, the purpose or intended use of the invention, rather than any distinct definition of any of the claimed invention's limitations, then the preamble is not considered a limitation and is of no significance to claim construction.** *Pitney Bowes, Inc. v. Hewlett-Packard Co.,* 182 F.3d 1298, 1305, 51 USPQ2d 1161, 1165 (Fed. Cir. 1999). See also *Rowe v. Dror,* 112 F.3d 473, 478, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997) ("where a patentee defines a structurally complete invention in the claim body and uses the preamble only to state a purpose or intended use for the invention, the preamble is not a claim limitation")... (Emphasis added)

6. Attention is directed to MPEP 2111. As stated therein:

During patent examination, the pending claims must be "given their broadest reasonable interpretation consistent with the specification." The Federal Circuit's *en banc* decision in *Phillips v. AWH Corp.*, 415 F.3d 1303, 1316, 75 USPQ2d 1321, 1329 (Fed. Cir. 2005) expressly recognized that the USPTO employs the "**broadest reasonable interpretation**" standard:

> The Patent and Trademark Office ("PTO") determines the scope of claims in patent applications not solely on the basis of the claim language, but upon giving claims their broadest reasonable construction "in light of the specification as it would be interpreted by one of ordinary skill in the art." *In re Am. Acad. of Sci. Tech. Ctr.*, 367 F.3d 1359, 1364[, 70 USPQ2d 1827, 1830] (Fed. Cir. 2004). Indeed, the rules of the PTO require that application claims must "conform to the invention as set forth in the remainder of the specification and the terms and phrases used in the claims must find clear support or antecedent basis in the description so that the meaning of the terms in the claims may be ascertainable by reference to the description." 37 CFR 1.75(d)(1). (Emphasis added).

Claim Rejections - 35 USC § 103

7. 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.

8. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Standard for Obviousness.

9. The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

10. Attention is directed to *In re Jung*, 98 USPQ2d 1174, 1178 (Fed. Cir. 2011) wherein is stated:

> There has never been a requirement for an examiner to make an on-the-record claim construction of every term in every rejected claim and to explain every possible difference between the prior art and the claimed invention in order to make out a *prima facie* rejection. This court declines to create such a burdensome and unnecessary requirement. "[Section 132] does not mandate that in order to establish prima facie anticipation, the PTO must explicitly preempt every possible response to a section 102 rejection. Section 132 merely ensures that an applicant at least be informed of the broad statutory basis for the rejection of his claims, so that he may determine what the issues are on which he can or should produce evidence." *Chester,* 906 F.2d at 1578 (internal citation omitted). As discussed above, all that is required of the office to meet its *prima facie* burden of production is to set forth the statutory basis of the rejection and the reference or references relied upon in a sufficiently articulate and informative manner as to meet the notice requirement of § 132. As the statute itself instructs, the examiner must "notify the applicant," "stating the reasons for such rejection," "together with such information and references as may be useful in judging the propriety of continuing prosecution of his application." 35 U.S.C. § 132.

11. Attention is directed to the decision in *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385 (U.S. 2007):

> When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense.

12. It is further noted that prior art is not limited to the four corners of the documentary prior art being applied. Prior art includes both the specialized understanding of one of ordinary skill in the art, and the common understanding of the layman. It includes "background knowledge

possessed by a person having ordinary skill in the art. . . [A] court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *KSR* at 1396.

13. Suggestion, teaching or motivation does not have to be explicit and "may be found in any number of sources, including common knowledge, the prior art as a whole or the nature of the problem itself" *Pfizer, Inc. v. Apotex, Inc.* 480 F.3d 1348, 82 USPQ2d 1321 (Fed. Cir. 2007) citing *Dystar Textilfarben GMBH v. C. H. Patrick Co.*, 464 F.3d 1356 (Fed. Cir. 2006).

Admissions as Prior Art.

14. Attention is directed to MPEP 706.02 [R-07.2015]:

> **III. RELIANCE ON ADMITTED PRIOR ART IN SUPPORT OF REJECTION**
> A statement by an applicant in the specification or made during prosecution identifying the work of another as "prior art" is an admission which can be relied upon for both anticipation and obviousness determinations, regardless of whether the admitted prior art would otherwise qualify as prior art under the statutory categories of 35 U.S.C. 102. *Riverwood Int'l Corp. v. R.A. Jones & Co.*, 324 F.3d 1346, 1354, 66 USPQ2d 1331, 1337 (Fed. Cir. 2003); *Constant v. Advanced Micro-Devices Inc.*, 848 F.2d 1560, 1570, 7 USPQ2d 1057, 1063 (Fed. Cir. 1988). See MPEP § 2129 for discussion on admissions as prior art. Where the admitted prior art anticipates the claim but does not qualify as prior art under any of the paragraphs of 35 U.S.C. 102, the claim may be rejected as being anticipated by the admitted prior art without citing to 35 U.S.C. 102.

15. Attention is directed to MPEP 2129 [R-08.2012], Admissions as Prior Art, which states in part:

> **I. ADMISSIONS BY APPLICANT CONSTITUTE PRIOR ART**
> A statement by an applicant in the specification or made during prosecution identifying the work of another as "prior art" is an admission **>which can be relied upon for both anticipation and obviousness determinations, regardless of whether the admitted prior art would otherwise qualify as prior art under the statutory categories of 35 U.S.C. 102. *Riverwood Int'l Corp. v. R.A. Jones & Co.*, 324 F.3d 1346, 1354, 66 USPQ2d 1331, 1337 (Fed. Cir. 2003); *Constant v. Advanced Micro-Devices Inc.*, 848 F.2d 1560, 1570, 7 USPQ2d 1057, 1063 (Fed. Cir. 1988).

16. Attention is also directed to *Ex parte Shirley*, (BPAI, 2009) Appeal No. 2009002352, which, at pages 21 and 26, states:

> The Specification's omission of the term "prior art" and inclusion of the prior-art disclaimer may initially appear to indicate that Appellants do not consider the single-step soft bake process to constitute prior art. To place these latter, contraindicative factors into the proper context though, we note that patent-application drafters regularly endeavor to avoid the indiscriminate or imprudent use of the descriptive label, "prior art." *See Riverwood Intern. Corp. v. R.A. Jones & Co., Inc.*, 324 F.3d 1346, 1354 (Fed. Cir. 2003) (citing *In re Fout*, 675 F.2d 297, 300 (CCPA 1982)) for the proposition that "section 102 is not the only source of ... prior art. Valid prior art may be created by the admissions of the parties"); *In re Nomiya*, 509 F.2d 566, 571 (CCPA 1975) (holding that an Applicant's labeling of certain figures as "prior art," *ipsissimis verbis*, constituted an admission that the pictured subject matter was prior art relative to Applicant's invention); MPEP § 21294 (instructing that "the examiner must determine whether the subject matter identified as 'prior art' is applicant's own work, or the work of another. In the absence of another credible explanation, examiners should treat such subject matter as the work of another").
>
> ***
>
> The Specification's omission of the term "prior art" and inclusion of the boilerplate prior-art disclaimer do not change our conclusion. In view of the record as a whole, these factors are ineffective in shielding Appellants from having their prior-art admissions treated as such.

17. Claims 82-84 and 87-100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 8,969,046 B2 (Van England et al.), US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.).

18. Applicant, at page 2 of the as-filed disclosure, under "BACKGROUND OF THE INVENTION," teaches that various methods, including immunochemical assays for detecting human hemoglobin in fecal sample was known in the art. As stated therein:

> 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 perioxidase 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 protpoporphyrin 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. (Emphasis added)

19. Applicant, page 5 of the disclosure, teaches that methods for detection of colorectal carcinoma based upon determining methylation of a number of different genes, including panels of genes, is disclosed in WO 2008/084219 A1 (Van England et al.).

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

20. Applicant, at page 17, third paragraph, admits that methods for isolating DNA are known in the art. As asserted to therein:

> 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 utilized. 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). (Emphasis added)

21. Applicant, at page 18, third paragraph, admits that kits for amplifying DNA in stool samples are commercially available. As admitted to therein:

> 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 TM (Biomerieux) and nucleic acid purification kits manufactured by Macherey Nagel.

22. Applicant, at page 19, further admits that kits for isolating DNA from fecal samples are commercially available. As asserted to therein:

> 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 1 ml 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. (Emphasis added)

23. Applicant, at page 21, last paragraph, bridging to page 22 of the as-filed disclosure admits that numerous methods for assessing methylation are known in the art, including methods that comprise polymerase chain reaction (PCR), which is recognized as a form of amplifying DNA. Also admitted to therein is the use of chemical reagents so to modify the DNA prior to amplification was also well known in the art. As admitted to therein:

> **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 diferences 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), 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. (Emphasis added)

24. Applicant, at page 24, third paragraph, admits that methods for performing MSP, wherein one employs primer pairs designed to distinguish methylated DNA from unmethylated DNA, was known in the art. As admitted to therein:

> 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 example, bisulphite ions modify non-methylated cytosine bases, changing them to uracil bases. Uracil bases hybridize to adenine bases under hybridization conditions. (Emphasis added)

25. The above showing is deemed to meet a limitation of claims 95, 96, and 100.

26. Applicant, at page 26, last paragraph, bridging to page 27, of the as-filed disclosure further asserts:

> 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. (Emphasis added)

27. Van England et al., have been found to teach determination of methylation of a number of different genes, including NDRG4 (elected species in claim 99). As stated in the abstract:

> A method of detecting a predisposition to, or the incidence of, cancer in a sample comprises detecting an epigenetic change in at least one gene selected from an **NDRG4**/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRPl, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNEl, S0X17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, cancer. (Emphasis added)

28. The above showing is deemed to meet a limitation of claims 95-99.

29. A review of the disclosure fails to find where applicant has admitted that using a concentration of 10 ng/ml of hemoglobin in stool as a threshold value was known in the art.

30. Yee et al., paragraph [0041], teaches setting a 10 ng/ml of hemoglobin in blood as a threshold value. Such a showing is deemed to meet a limitation of claims 87-94.

31. Neither the disclosure nor Yee et al., have been found to teach freezing a portion of a fecal sample.

32. Grow et al., abstract, teach the preparation of a fecal sample for immunoassay, which includes determining if human hemoglobin is present. As disclosed therein:

> A method for preparing a fecal sample for immunoassay testing comprising the steps of dispersing **a sample of from 1 to less than 10 wt. % of a stool sample** in an aqueous fecal test solution formulated with one or more preservatives, analyte stabilizing agents and endogenous interference reducing agents. The fecal solids are then permitted to settle to form a liquid phase substantially free from fecal solids, and the clarified liquid phase is removed to provide a test sample free from fecal solids. **The fecal test solutions contain suitable stool stabilizers such as buffers** and antimicrobial agents, analyte protecting agents such as proteolytic, reductive or oxidative enzyme inhibitors, endogenous assay interfering enzyme inhibitors such as a reducing agent, and non-specific binding inhibitors such as animal proteins. **The stool sample should be fresh or be fresh frozen** and thawed immediately before dispersion in the buffer solution. The sample is suitable for any solid-phase immunoassay determination of a fecal sample analyte. A method for

> determining analyte in the stool sample comprises conjugating anti-analyte antibody adhered to a insoluble support with analyte in the clarified sample, and determining the presence and extent of such conjugation. For determining human hemoglobin in a sample of human stool, the aqueous fecal test solution preferably contains a proteinase inhibitor, formaldehyde and an animal albumin. (Emphasis added)

33. The fact that only 1 to less than 10% of the sample is being used is deemed to meet a limitation of claim 82, step b). The fact that a buffer is being used is deemed to meet a limitation of claim 82, step c). The fact that the sample can be frozen is deemed to meet a limitation of claim 83.

34. As evidenced above, Grow et al., teach preparation of a fecal sample for immunological testing, not analysis of nucleic acids contained therein.

35. Shuber et al., teach methods for stabilizing biological samples for nucleic acid analysis. As seen at paragraph [0009], the sample can be that of a patient's fecal sample. Shuber et al., paragraph [0031], teach:

> However, according to the invention, it may be particularly important to preserve sample DNA for purification, especially when looking for early indicia of diseases (e.g., indicia of adenomas or early stage cancers that may be present in less than about 1%, or about 0.1% or less of human genomes isolated from a stool sample). A common method to insure that DNA remains stable is to freeze stool samples as quickly as possible after collection, or to receive samples in centralized testing labs as quickly as possible.

36. Shuber et al., Example 2, teach the benefits of treating a stool sample with a nucleic acid stabilization solution. Such a showing is deemed to meet a limitation of claims 82 and 83.

37. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to analyze fecal samples for both blood (hemoglobin) as well as for the NDRG4 gene. It would also have bene obvious to collect separate portion of a sample and to treat each in a manner that would either preserve the protein target(s) (Grow et al.) or the nucleic acid target(s) (Shuber et al.).

38. In view of the intense interest in detection of colorectal carcinoma in an individual, said ordinary artisan would have been amply motivated. And in view of the well-developed state of the art, and the fact that one would be using the disclosed methods for the same purpose, said ordinary artisan would have had a most reasonable expectation of success.

39. In view of the above analysis and in the absence of convincing evidence to the contrary, claims 82-84 and 87-100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 8,969,046 B2 (Van England et al.), US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.).

Conclusion

40. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Bradley L. Sisson whose telephone number is (571)272-0751. The examiner can normally be reached on Monday to Thursday, from 6:30 AM to 5 PM..

41. Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

42. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on 571-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

43. 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.

/Bradley L. Sisson/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost	
	Examiner Bradley L Sisson	Art Unit 1634	Page 1 of 1

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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*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.

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✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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CLAIMS

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

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019							
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
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CPC - Searched*		
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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
EAST (see transcript for databases and search terms)	06/23/2019	B. L. S.
Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
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U.S. PATENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
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Note: Submission of copies of U.S. Patents and published U.S. Patent Applications is not required.

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.L.S./

Examiner Signature	/Bradley L. Sisson/	Date Considered	06/23/2019

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 (Use as many sheets as necessary)				Complete if Known	
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				Art Unit	1634
				Examiner Name	SISSON
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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]
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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.L.S./

Examiner Signature	/Bradley L. Sisson/	Date Considered	06/23/2019

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NONPATENT LITERATURE DOCUMENTS			
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		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.	
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		ITZKOWITZ et al., A simplified, noninvasive stool DNA test for colorectal cancer detection. Am J Gastroenterol. 2008 Nov;103(11):2862-70	
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		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	
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		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	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.L.S./

Examiner Signature	/Bradley L. Sisson/	Date Considered	06/23/2019

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 (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	4	of	5	Attorney Docket Number	EXCTD-35239/US-4/CON

NONPATENT LITERATURE DOCUMENTS			
		VILLAR-GAREA et al., DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Curr Drug Metab. 2003 Feb;4(1):11-31	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.L.S./

Examiner Signature	/Bradley L. Sisson/	Date Considered	06/23/2019

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 (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
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				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
Sheet	5	of	5	*Attorney Docket Number*	EXCTD-35239/US-4/CON

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.

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)	2019-05-23
Name/Print	Mary Ann D. Brow	Registration Number	42363

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

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	53	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:50
S2	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:51
S3	9	(hemoglobin haemoglobin) with concentration same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:18
S4	260	DNA same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:35
S5	2	"20080227208"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:34
S6	1	"20080227208" and "10 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:35
S7	11	(hemoglobin haemoglobin) with "5 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:38

EAST Search History (Interference)

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BIB DATA SHEET

CONFIRMATION NO. 1874

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/634,607	06/27/2017 RULE	435	1634	EXCTD-35239/US-4/CON

APPLICANTS

EXACT SCIENCES DEVELOPMENT COMPANY, LLC, Madison, WI;

INVENTORS

Joost Louwagie, Dornach, SWITZERLAND;

**** CONTINUING DATA ***************************

This application 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 ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

07/12/2017

Foreign Priority claimed	❑ Yes ☑ No		STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
35 USC 119(a-d) conditions met	❑ Yes ☑ No	❑ Met after Allowance				
Verified and Acknowledged	/BRADLEY L SISSON/ Examiner's Signature	Initials	SWITZERLAND	0	~~19~~ 17	~~2~~ 1

ADDRESS

Casimir Jones, S.C.
2275 Deming Way Ste 310
Middleton, WI 53562
UNITED STATES

TITLE

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

FILING FEE RECEIVED		
1740	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other _____ ❑ Credit

To: docketing@casimirjones.com,pto.correspondence@casimirjones.com,
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Cc: PAIR_eOfficeAction@uspto.gov
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Jun 27, 2019 04:00:58 AM

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Application	Document	Mailroom Date	Attorney Docket No.
15634607	CTNF	06/27/2019	EXCTD-35239/US-4/CON
	892	06/27/2019	EXCTD-35239/US-4/CON
	1449	06/27/2019	EXCTD-35239/US-4/CON

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PATENT
Attorney Docket No.: **EXCTD-35239.304**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

RESPONSE TO OFFICE ACTION MAILED 06/27/2019

EFS WEB FILED
Commissioner for Patents
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CERTIFICATE OF EFS WEB TRANSMISSION UNDER 37 C.F.R. § 1.8

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

Dated: September 27, 2019 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

Examiner Sisson:

This paper is responsive to the Office Action mailed 06/27/2019, with response due by 09/27/2019. 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-35239.304**. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Remarks begin on Page 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.	:	13/147,570
Applicant	:	Joost Louwagie
Filed	:	March 12, 2012
Title	:	METHODS FOR DETECTING COLORECTAL CANCER
TC/A.U.	:	1634
Examiner	:	Bradley L. Sisson
Docket No.	:	5545-00036
Conf. No.	:	4272

INVENTOR'S DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

I, Joost Louwagie, hereby declare and state that:

1. I am the sole inventor of the methods of determining a predisposition to, or the incidence of, colorectal cancer in a fecal sample from a human patient, comprising (a) detecting human blood in the fecal sample; and (b) detecting methylation of one or more CpG dinucleotide motifs in the promoter of NDRG4 in DNA contained within the fecal sample, as disclosed and presently claimed in the above-captioned U.S. Patent Application No. 13/147,570 (hereinafter "the '570 Non-Provisional Application"), filed on March 12, 2012, which is the U.S. National Stage of International Application PCT/GB2010/000180, filed on February 3, 2010.

2. I am the sole inventor of the methods of determining a predisposition to, or the incidence of, colorectal cancer in a fecal sample from a human patient, comprising (a) detecting human blood in the fecal sample; and (b) detecting methylation of one or more CpG dinucleotide motifs in the promoter of NDRG4 in DNA contained within the fecal sample as disclosed and claimed in U.S. Provisional Application No. 61/149,581, filed on February 3, 2009 (hereinafter "the '581 Provisional Application") to which the '570 Non-Provisional Application claims the benefit of priority.

3. I am a co-inventor of the subject matter disclosed in International Application PCT/GB2008/000056, which lists as inventors Manon Van Engeland, Manon Adrian De Bruine, Arjan Griffioen, myself, and Katja Bierau and was published as WO 2008/084219 on July 17, 2008 (hereinafter the '219 Int'l Published App"). In so far as the '219 Int'l Published App is asserted to disclose methods of determining a predisposition to, or the incidence of, colorectal cancer in a fecal sample from a human patient, comprising detecting methylation of one or more CpG dinucleotide motifs in the promoter of NDRG4 in DNA contained within the fecal sample, these methods were invented by me and no other.

4. The subject matter disclosed in the '570 Non-Provisional Application and the '581 Provisional Application and related to methods of determining a predisposition to, or the incidence of, colorectal cancer in a fecal sample from a human patient, comprising (a) detecting human blood in the fecal sample; and (b) detecting methylation of one or more CpG dinucleotide motifs in the promoter of NDRG4 in DNA contained within the fecal sample was invented solely by me and no others.

5. The listed co-inventors of the '219 Int'l Published App, other than myself, namely Manon Van Engeland, Manon Adrian De Bruine, Arjan Griffioen, and Katja Bierau, did not make an inventive contribution to the subject matter claimed in the '570 Non-Provisional Application and the '581 Provisional Application and therefore are not co-inventors of the subject matter claimed in the '570 Non-Provisional Application and the '581 Provisional Application.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any parent resulting therefrom.

By _____ Date: July 9, 2016

Joost Louwagie

Electronic Acknowledgement Receipt

EFS ID:	37306987
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	27-SEP-2019
Filing Date:	27-JUN-2017
Time Stamp:	18:05:34
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2019-09-27_35239-304_ROA.pdf	135586 90d1a9250b01cccde1cf6f84dd1b39e5f90dc391	yes	5

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Applicant Arguments/Remarks Made in an Amendment		2	5	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
Warnings:					
Information:					
2	Affidavit-traversing rejectns or objectns rule 132	2019-09-27_Declaration_Louwagie.pdf	1558263 20c82e18f53574d9cd095613da6aed68a77077f4	no	2
Warnings:					
Information:					
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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 a 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.

REMARKS

Claims 82-84 and 87-100 are pending and under examination. In the Office Action mailed 06/27/2019 ("Office Action"), claims 82-84 and 87-100 were rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Applicant's admissions in view of US 8,969,046 B2 ("Van England U.S. Patent"), US 2008/0227208 A1 ("Yee"), US 5,198,365 ("Grow"), and US 2008/0124714 A1 ("Shuber").

The Van Engeland U.S. Patent is the national stage of PCT/GB2008/000056, which was published as WO 2008/084219 on July 17, 2008 (hereinafter "the Van Engeland Published PCT"). The present application is a continuation of U.S. Application Ser. No. 13/147,570 (the "'570 Application"), which claims priority to U.S. Provisional Application No. 61/149,581, filed on February 3, 2009. The Van Engeland Published PCT is substantially identical to the Van Engeland U.S. Patent in regard to the information disclosed therein related to detecting methylation of NDRG4 as an indication of colorectal cancer.

Applicant has enclosed herewith an Inventor's Declaration Under 37 C.F.R. § 1.132 by inventor Joost Louwagie, dated July 9, 2016, and made of record in U.S. Pat. Application Ser. No. 13/147,570, parent to the present application. In the Declaration, inventor Louwangie indicates that the Van Engeland Published PCT does not disclose invention "by another," as required under 35 U.S.C. § 102(a). Consequently, the presently cited Van Engeland U.S. Patent is also not prior art under 35 U.S.C. § 102(a) to the claims of the present Application. As will be explained below, the remaining cited references, Yee, Grow, and Shuber, fail to establish prima facie obviousness of Applicant's claims 82-84 and 87-100.

The Examiner points to Yee as teaching a 10 ng/ml of hemoglobin as a threshold value, to Grow as teaching preparation of a fecal sample for immunoassay, and to Shuber as teaching treating a stool sample with a nucleic acid stabilizing solution. However, none of Yee, Grow, or Shuber teach or suggest analysis of a single collected fecal sample for <u>both</u> hemoglobin and nucleic acid, much less testing hemoglobin in combination with the *NDRG4* nucleic acid marker of Applicant's elected species. Further, the combination of references does not point to collecting and processing a fecal sample in the manner set forth in Applicant's claims 82-84 and 87-100, in which the sample is divided and the portions are separately treated to preserve blood

proteins in a removed portion and to stabilize the remaining portion, *e.g.*, for extraction of DNA for analysis.

In making the rejection, the Examiner states that "In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to analyze fecal samples for both blood (hemoglobin) as well as for the NDRG4 gene." (Office Action, ¶37). The Examiner further asserts that "In view of the intense interest in detection of colorectal carcinoma in an individual, said ordinary artisan would have been amply motivated. And in view of the well-developed state of the art, and the fact that one would be using the disclosed methods for the same purpose, said ordinary artisan would have had a most reasonable expectation of success." (Office Action, ¶38). The Examiner provides no additional analysis or argument in support of making the combination.

A valid prima facie case requires the Office to present "articulated reasoning with some rational underpinning", *KSR*, 550 U.S. at 418 (citing *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)), that one of skill in the art would have been motivated to combine the elements to yield the claimed combination and that one would have done so having a reasonable expectation of successfully carrying out the claimed combination. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991). A failure to properly establish either a motivation to combine references or a reasonable expectation of success in making the combination precludes finding a prima facie case of obviousness, and, without more, such failure entitles the an Applicant to allowance of the claims at issue. See MPEP § 2143. Thus, the Office must provide an objective reason why one of skill in the art *would have*, not merely *could have*, combined or modified the teachings of the cited art.

While *KSR* relaxed some of the formalism accompanying earlier decisions requiring a "teaching, suggestion, or motivation" to combine prior art references, it did not eliminate the requirement to anchor the nonobviousness calculus in an explanation of how a person of ordinary skill would select and apply the teachings of the cited art. See, e.g., *KSR*, 550 U.S. at 417. Rather, rejections on obviousness "cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning" *KSR*, 550 U.S. at 418 (citing *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)), that one of skill in the art would have been motivated to combine the elements to yield the claimed combination. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991). See MPEP § 2143.

In the present case, the Examiner has provided no such "articulated reasoning" to support a conclusion of prima facie obviousness. Instead, the Examiner has pointed only to "the intense interest in detection of colorectal carcinoma in an individual" as a basis for asserting that the skilled person would be motivated make the combination. This conclusory statement is not sufficient to establish prima facie obviousness of the instant claim over these references.

The teachings of these references enumerated in the Office Action are related to immunoassay analysis of fecal samples for occult blood/hemoglobin (Yee and Grow) or for stabilizing samples for nucleic acid analysis (Shuber). For hemoglobin immunoassays, Yee and Grow teach methods for collecting, storing, processing, and testing fecal samples that are completely different than the methods taught by Shuber for nucleic acid analysis. Consequently, analyzing a fecal sample for both blood/hemoglobin and nucleic acid using the methods disclosed by Yee, Grow, and Shuber would result in a significant increase in work and cost compared to using any one of the methods alone.

Notably, however, neither the references nor the Examiner identify any deficits blood/hemoglobin testing of fecal samples that are curable by the addition of Shuber's nucleic acid testing, nor vice versa. The references thus provide no suggestion that combining these methods of analysis, as urged by the Examiner, might offer any particular improvement or benefit over use of one testing method alone. Rather, the skilled person considering the cited art - *with or without Van Engeland* - could only guess at whether using both blood/hemoglobin testing and nucleic acid testing on a fecal sample *might* produce some beneficial result.

Although the Examiner asserts that the skilled person would have been amply motivated to make the combination by "intense interest in detection of colorectal carcinoma in an individual," the Examiner has not shown why, based on the cited art, a skilled person would believe that testing fecal samples using both blood/hemoglobin testing and nucleic acid testing would be expected to offer any benefit with respect to detection of colorectal carcinoma at all, much less a benefit of sufficient importance or magnitude to offset the increase in costs and labor of running multiple, different testing methods on each sample.

While not acquiescing that other requirements for establishing prima facie obviousness have been met, for the reasons set forth above, Applicant submits that prima facie obviousness of claims 82-84 and 87-100 over Yee, Grow, and Shuber - *with or without Van Engeland* - has not been established for at least the reason that the Examiner has relied on conclusory statements and

has failed to establish as required that one of skill in the art would have been motivated to combine the elements to yield the claimed combination. Applicant therefore respectfully requests that the rejection of claims 82-84 and 87-100 be withdrawn.

CONCLUSION

For the reasons set forth above, 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: September 27, 2019

/Mary Ann D. Brow /

Mary Ann D. Brow
Registration No. 42,363

CASIMIR JONES, S.C.
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Middleton, Wisconsin 53562
608.662.1277

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

72960 7590 01/21/2020

Casimir Jones, S.C.
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EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
01/21/2020	ELECTRONIC

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

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

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

docketing@casimirjones.com
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Office Action Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Bradley L Sisson	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 09/27/2019.

☐ 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) 82-84 and 87-100 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) 8-84 and 87-100 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)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Claim Interpretation

2. Attention is directed to MPEP 904.01 [R-08.2012].

> The breadth of the claims in the application should always be carefully noted; that is, the examiner should be fully aware of what the claims do not call for, as well as what they do require. During patent examination, the claims are given the broadest reasonable interpretation consistent with the specification. See *In re Morris*, 127 F.3d 1048, 44 USPQ2d 1023 (Fed. Cir. 1997). See MPEP § 2111 - § 2116.01 for case law pertinent to claim analysis.

3. It is noted with particularity that narrowing limitations found in the specification cannot be inferred in the claims where the elements not set forth in the claims are linchpin of patentability. *In re Philips Industries v. State Stove & Mfg. Co, Inc.*, 186 USPQ 458 (CA6 1975). While the claims are to be interpreted in light of the specification, it does not follow that limitations from the specification may be read into the claims. On the contrary, claims must be interpreted as broadly as their terms reasonably allow. See *Ex parte Oetiker,* 23 USPQ2d 1641 (BPAI, 1992). In added support of this position, attention is directed to MPEP 2111 [R-11.2013], where, citing *In re Prater*, 415 F.2d 1393, 1404-05, 162 USPQ 541, 550-51 (CCPA 1969), is stated:

> The court explained that "reading a claim in light of the specification, to thereby interpret limitations explicitly recited in the claim, is a quite different thing from 'reading limitations of the specification into a claim,' to thereby narrow the scope of the claim by implicitly adding disclosed limitations which have no express basis in the claim." The