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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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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

City	Dornach	Country of Residence [i]	CH

Mailing Address of Inventor:

Address 1	441 Charmany Drive		
Address 2			
City	Madison	**State/Province**	WI
Postal Code	53719	**Country** [i]	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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	Application Number	
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	

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.

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	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:			
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. 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

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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 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.

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

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(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.

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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

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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.

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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

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(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
Filing Date:	
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	-
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Information:					
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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Input Set:

Output Set:

Started: 2017-06-27 16:25:13.636
Finished: 2017-06-27 16:25:14.388
Elapsed: 0 hr(s) 0 min(s) 0 sec(s) 752 ms
Total Warnings: 39
Total Errors: 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	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	
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Total Indep	21		0		0	
Total Depend	80	↵	0	↵	0	↵
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



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	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
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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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
Registration No. 42,363

CASIMIR JONES, S.C.
2275 Deming Way, Suite 310
Middleton, Wisconsin 53562
608.662.1277

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
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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		

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New International Application Filed with the USPTO as a Receiving Office
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~~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.

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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.

Exact Sciences Ex. 2003, Page 125 of 1394

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.

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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

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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

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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 ~~-2O0C~~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).

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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.

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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~~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

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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

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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.

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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

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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~~0C~~°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.

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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

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(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

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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.

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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

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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).

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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.

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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

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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

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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

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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

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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:

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(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.

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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.

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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

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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.

Exact Sciences Ex. 2003, Page 226 of 1394

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	

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	Application Size Fee (37 CFR 1.16(s))									
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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
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  Joost Louwagie

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15634607	APP.FILE.REC	10/17/2017	EXCTD-35239/US-4/CON
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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

PUBLICATION NOTICE

OC000000094862231

Title:METHODS FOR DETECTING COLORECTAL CANCER

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

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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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Fax.: 608-662-1276

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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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:

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/AIA/01 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.

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

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		

Corrected ADS 1.0

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

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

Document Description: Application Data Sheet to update/correct info
Doc Code: ADS.CORR

****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		

Corrected ADS 1.0

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		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.
<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
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

OC000000101215749

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

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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 Gabriel E. Cleveland	Date Jan. 11, 2018
Name Gabriel E. Cleveland	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)
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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)
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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

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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
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

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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
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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 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	-								
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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 RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/634,607	06/27/2017	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	❑ Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
35 USC 119(a-d) conditions met	❑ Yes ☑ No					
Verified and Acknowledged	/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

BIB (Rev. 05/07).

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.

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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

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
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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~~ 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

PTO/AIA/14 (11-15)
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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~~ 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.

PTO/AIA/14 (11-15)
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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~~ 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.

PTO/AIA/14 (11-15)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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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~~ 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.

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
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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~~ 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.

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
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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~~ 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.

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.	
		HAMMER, Human hybirds. Sci Am. 2013 May;308(5):66-71	
		ITZKOWITZ et al., A simplified, noninvasive stool DNA test for colorectal cancer detection. Am J Gastroenterol. 2008 Nov;103(11):2862-70	
		KARL et al., Improved diagnosis of colorectal cancer using a combination of fecal occult blood and novel fecal protein markers. Clin Gastroenterol Hepatol. 2008 Oct;6(10):1122-8	
		LAIRD, The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003 Apr;3(4):253-66	
		LENHARD et al., Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. Clin Gastroenterol Hepatol. 2005 Feb;3(2):142-9	
		LEVI et al., A quantitative immunochemical faecal occult blood test is more efficient for detecting significant colorectal neoplasia than a sensitive guaiac test. Aliment Pharmacol Ther. 2006 May 1;23(9):1359-64	
		MULDER et al., Tumor pyruvate kinase isoenzyme type M2 and immunochemical fecal occult blood test: performance in screening for colorectal cancer. Eur J Gastroenterol Hepatol. 2007 Oct;19(10):878-82	
		REIN et al., Identifying 5-methylcytosine and related modifications in DNA genomes. Nucleic Acids Res. 1998 May 15;26(10):2255-64	
		SHASTRI et al., Comparison of an established simple office-based immunological FOBT with fecal tumor pyruvate kinase type M2 (M2-PK) for colorectal cancer screening: prospective multicenter study. Am J Gastroenterol. 2008 Jun;103(6):1496-504	
		SOMMER et al., Minimal homology requirements for PCR primers. Nucleic Acids Res. 1989 Aug 25;17(16):6749	

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
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				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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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) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	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) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	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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*	B	US-8969046-B2	03-2015	Van Engeland; Manon	C12Q1/6886	435/91.2
*	C	US-20080227208-A1	09-2008	Yee; Hsiao-Ching	G01N33/72	436/66
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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.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
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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
				Examiner Name	SISSON
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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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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
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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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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]

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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				Filing Date	06/27/2017
				First Named Inventor	JOOST
				Art Unit	1634
				Examiner Name	SISSON
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		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	

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	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.	
		HAMMER, Human hybirds. Sci Am. 2013 May;308(5):66-71	
		ITZKOWITZ et al., A simplified, noninvasive stool DNA test for colorectal cancer detection. Am J Gastroenterol. 2008 Nov;103(11):2862-70	
		KARL et al., Improved diagnosis of colorectal cancer using a combination of fecal occult blood and novel fecal protein markers. Clin Gastroenterol Hepatol. 2008 Oct;6(10):1122-8	
		LAIRD, The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003 Apr;3(4):253-66	
		LENHARD et al., Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. Clin Gastroenterol Hepatol. 2005 Feb;3(2):142-9	
		LEVI et al., A quantitative immunochemical faecal occult blood test is more efficient for detecting significant colorectal neoplasia than a sensitive guaiac test. Aliment Pharmacol Ther. 2006 May 1;23(9):1359-64	
		MULDER et al., Tumor pyruvate kinase isoenzyme type M2 and immunochemical fecal occult blood test: performance in screening for colorectal cancer. Eur J Gastroenterol Hepatol. 2007 Oct;19(10):878-82	
		REIN et al., Identifying 5-methylcytosine and related modifications in DNA genomes. Nucleic Acids Res. 1998 May 15;26(10):2255-64	
		SHASTRI et al., Comparison of an established simple office-based immunological FOBT with fecal tumor pyruvate kinase type M2 (M2-PK) for colorectal cancer screening: prospective multicenter study. Am J Gastroenterol. 2008 Jun;103(6):1496-504	
		SOMMER et al., Minimal homology requirements for PCR primers. Nucleic Acids Res. 1989 Aug 25;17(16):6749	

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	
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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).
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[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**.

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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CONFIRMATION NO. 1874

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/634,607	06/27/2017	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 35 USC 119(a-d) conditions met ❑ Yes ☑ No Verified and Acknowledged /BRADLEY L SISSON/ Examiner's Signature	❑ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		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

BIB (Rev. 05/07).

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

Jun 27, 2019 04:00:58 AM

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Casimir Jones, S.C.
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Middleton, WI 53562
UNITED STATES

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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
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: 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:					
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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 both 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 /

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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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
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 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

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, 87-98, and 100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.).

17. 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)

18. 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.

19. 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)

20. 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.

21. 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)

22. 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)

23. 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)

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

25. 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)

26. 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.

27. 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.

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

29. 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 an 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)

30. 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.

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

32. 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.

33. 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.

34. As evidenced above, both Shuber et al., and Grow et al., teach freezing the sample, be it for immunological testing or analysis of the nucleotide sequence of a gene, and the methylation thereof.

35. 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 a gene/sequence of interest. 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.).

36. 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.

37. In view of the above analysis and in the absence of convincing evidence to the contrary, claims 82-84, 87-98, and 100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.).

38. Claim 99 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.) as applied to claims 82-84, 87-98, and 100 above, and further in view of WO 2004/083399 A2 (Goggins et al.).

39. See above for the basis sf the rejection as it relates to the admissions of applicant and the teachings of Yee et al., Grow et al., and Shuber et al.

40. Neither admissions of applicant, nor the teachings of Yee et al., Grow et al., and Shuber et al., have been found to teach the genes listed in claim 99.

41. Goggins et al., in their abstract, teach:

> The present invention provides a method for detecting pancreatic carcinoma in a subject. The method includes contacting a nucleic acid-containing specimen from the subject with an agent that provides a determination of the methylation state of at least one gene or associated regulatory region of the gene and identifying aberrant methylation of regions

of the gene or regulatory region, wherein aberrant methylation is identified as being different when compared to the same regions of the gene or associated regulatory region in a subject not having the pancreatic carcinoma, thereby detecting pancreatic carcinoma in the subject.

42. Goggins et al., at page 8, teach:

> Genes associated with pancreatic carcinoma are listed in Tables 1-3 by way of example. Specifically, such genes include CDH3, reprimo, CLDN5, DR3, **FOXE1**, LDOCl, LHXl, NEFH, NPTX2, PIGll, SARP2, ST14, SMARCAl, TJP2, UCHLl, WNT7A, or a combination thereof. (Emphasis added)

43. Goggins et al., at paragraph [0042] (page 16), teach that the cells to be tested can be obtained from the colon. As stated therein:

> The test cell also can be **a component of a biological fluid**, for example, blood, lymph, cerebrospinal fluid, pancreatic juice, saliva, sputum, stool, urine, or ejaculate. If appropriate, the test cells also can be obtained by lavage, for example, for obtaining test cells from the **colon**, uterus, abdominal cavity, or the like, or using an aspiration procedure, for example, for obtaining a bone marrow sample. (Emphasis added)

44. Goggins et al., paragraph [0079], teach that the neoplasm can be that which occurs in the colon. As disclosed therein:

> A neoplasm creates an unstructured mass (a tumor) which can be either benign or malignant. For example, the neoplasm may be a head, neck, lung, esophageal, stomach, small bowel, **colon**, bladder, kidney, or cervical neoplasm. (Emphasis added)

45. 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 have modified the teachings of applicant, Yee et al., Grow et al., and Shuber et al., whereby the gene to be evaluated in terms of methylation was FOXE1 as it had already been associated with cancer.

46. In view of the well-developed state of the art, and the specific guidance to evaluate the methylation of this specific gene as it had been associated with cancer, said ordinary artisan would have been amply motivated to have evaluated same, using known methods of sample collection, and analysis.

47. In view of the above analysis and in the absence of convincing evidence to the contrary, claim 99 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.) as applied to claims 82-84, 87-98, and 100 above, and further in view of WO 2004/083399 A2 (Goggins et al.).

Conclusion

48. Objections and/or rejections which appeared in the prior Office action and which have not been repeated hereinabove have been withdrawn.

49. 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..

50. 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.

51. 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.

52. 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

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N	WO-2004083399-A2	09-2004	WO	GOGGINS, Michael G.	C12Q1/6827
	O					
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NON-PATENT DOCUMENTS

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

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

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
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	Bradley L Sisson	1634

✓	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	01/15/2020						
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CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020						
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	Art Unit
	Bradley L Sisson	1634

CPC - Searched*		
Symbol	Date	Examiner

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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
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Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.
EAST (see transcript for databases and search terms)	01/14, 15/2020	B. L. S.
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(54) Title: ABERRANTLY METHYLATED GENES IN PANCREATIC CANCER

(57) Abstract: The present invention provides a method for detecting pancreatic carcinoma in a subject. The method includes contacting a nucleic acid-containing specimen from the subject with an agent that provides a determination of the methylation state of at least one gene or associated regulatory region of the gene and identifying aberrant methylation of regions of the gene or regulatory region, wherein aberrant methylation is identified as being different when compared to the same regions of the gene or associated regulatory region in a subject not having the pancreatic carcinoma, thereby detecting pancreatic carcinoma in the subject.

WO 2004/083399 A2

ABERRANTLY METHYLATED GENES IN PANCREATIC CANCER

RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application Serial No. 60/454,614, filed March 17, 2003, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the regulation of gene expression and more specifically to a method of determining the DNA methylation status of CpG sites in a given locus and correlating the methylation status with the presence of a cell proliferative disorder.

BACKGROUND OF THE INVENTION

[0003] DNA methylases transfer methyl groups from the universal methyl donor S-adenosyl methionine to specific sites on the DNA. Several biological functions have been attributed to the methylated bases in DNA. The most established biological function for methylated DNA is the protection of DNA from digestion by cognate restriction enzymes. The restriction modification phenomenon has, so far, been observed only in bacteria. Mammalian cells, however, possess a different methylase that exclusively methylates cytosine residues that are 5' neighbors of guanine (CpG). This modification of cytosine residues has important regulatory effects on gene expression, especially when involving CpG rich areas, known as CpG islands, located in the promoter regions of many genes.

[0004] Methylation has been shown by several lines of evidence to play a role in gene activity, cell differentiation, tumorigenesis, X-chromosome inactivation, genomic imprinting and other major biological processes (Razin, A., H., and Riggs, R.D. eds. in DNA Methylation Biochemistry and Biological Significance, Springer-Verlag, New York, 1984). In eukaryotic cells, methylation of cytosine residues that are immediately 5' to a guanosine, occurs predominantly in CG poor regions (Bird, A., Nature, 321:209,

1986). In contrast, CpG islands remain unmethylated in normal cells, except during X-chromosome inactivation (Migeon, et al., supra) and parental specific imprinting (Li, et al., Nature, 366:362, 1993) where methylation of 5' regulatory regions can lead to transcriptional repression. De novo methylation of the Rb gene has been demonstrated in a small fraction of retinoblastomas (Sakai, et al., Am. J. Hum. Genet., 48:880, 1991), and recently, a more detailed analysis of the VHL gene showed aberrant methylation in a subset of sporadic renal cell carcinomas (Herman, et al., Proc. Natl. Acad. Sci., U.S.A., 91:9700, 1994). Expression of a tumor suppressor gene can also be abolished by de novo DNA methylation of a normally unmethylated CpG island (Issa, et al., Nature Genet., 7:536, 1994; Herman, et al., supra; Merlo, et al., Nature Med., 1:686, 1995; Herman, et al., Cancer Res., 56:722, 1996; Graff, et al., Cancer Res., 55:5195, 1995; Herman, et al., Cancer Res., 55:4525, 1995).

[0005] Human cancer cells typically contain somatically altered nucleic acid, characterized by mutation, amplification, or deletion of critical genes. In addition, the nucleic acid from human cancer cells often displays somatic changes in DNA methylation (E.R. Fearon, et al., Cell, 61:759, 1990; P.A. Jones, et al., Cancer Res., 46:461, 1986; R. Holliday, Science, 238:163, 1987; A. De Bustros, et al., Proc. Natl. Acad. Sci., USA, 85:5693, 1988); P.A. Jones, et al., Adv. Cancer Res., 54:1, 1990; S.B. Baylin, et al., Cancer Cells, 3:383, 1991; M. Makos, et al., Proc. Natl. Acad. Sci., USA, 89:1929, 1992; N. Ohtani-Fujita, et al., Oncogene, 8:1063, 1993). However, the precise role of abnormal DNA methylation in human tumorigenesis has not been established. Aberrant methylation of normally unmethylated CpG islands has been described as a frequent event in immortalized and transformed cells, and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers. In the development of colorectal cancers (CRC), a series of tumor suppressor genes (TSG) such as APC, p53, DCC and DPC4 are inactivated by mutations and chromosomal deletions. Some of these alterations result from a chromosomal instability phenotype described in a subset of CRC. Recently, an additional pathway has been shown to be involved in a familial form of CRC, hereditary non-polyposis colorectal cancer. The cancers from these patients show a characteristic mutator phenotype which causes microsatellite

instability (MI), and mutations at other gene loci such as TGF-β-RII (Markowitz et al., Science, 268(5215):1336-8, 1995) and BAX. This phenotype usually results from mutations in the mismatch repair (MMR) genes hMSH2 and hMLH1. A subset of sporadic CRC also show MI, but mutations in MMR genes appear to be less frequent in these tumors.

[0006] Another molecular defect described in CRC is CpG island (CGI) methylation. CGIs are short sequences rich in the CpG dinucleotide and can be found in the 5' region of about half of all human genes. Methylation of cytosine within 5' CGIs is associated with loss of gene expression and has been seen in physiological conditions such as X chromosome inactivation and genomic imprinting. Aberrant methylation of CGIs has been detected in genetic diseases such as the fragile-X syndrome, in aging cells and in neoplasia. About half of the tumor suppressor genes which have been shown to be mutated in the germline of patients with familial cancer syndromes have also been shown to be aberrantly methylated in some proportion of sporadic cancers, including Rb, VHL, p16, hMLH1, and BRCA1. TSG methylation in cancer is usually associated with (Antequera, et al., Proc. Natl. Acad. Sci. USA, 90:11995-11999, 1993) lack of gene transcription and (Baylin, et al., Adv. Cancer Res., 72:141-196, 1998) absence of coding region mutation. Thus it has been proposed that CGI methylation serves as an alternative mechanism of gene inactivation in cancer.

[0007] The causes and global patterns of CGI methylation in human cancers remain poorly defined. Aging could play a factor in this process because methylation of several CGIs could be detected in an age-related manner in normal colon mucosa as well as in CRC. In addition, aberrant methylation of CGIs has been associated with the MI phenotype in CRC as well as specific carcinogen exposures. However, an understanding of aberrant methylation in CRC has been somewhat limited by the small number of CGIs analyzed to date. In fact, previous studies have suggested that large numbers of CGIs are methylated in immortalized cell lines, and it is not well understood whether this global aberrant methylation is caused by the cell culture conditions or whether they are an integral part of the pathogenesis of cancer.

[0008] Most of the methods developed to date for detection of methylated cytosine depend upon cleavage of the phosphodiester bond alongside cytosine residues, using either methylation-sensitive restriction enzymes or reactive chemicals such as hydrazine which differentiate between cytosine and its 5-methyl derivative. Genomic sequencing protocols which identify a 5-MeC residue in genomic DNA as a site that is not cleaved by any of the Maxam Gilbert sequencing reactions have also been used, but still suffer disadvantages such as the requirement for large amount of genomic DNA and the difficulty in detecting a gap in a sequencing ladder which may contain bands of varying intensity.

[0009] Mapping of methylated regions in DNA has relied primarily on Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences which contain one or more methylated CpG sites. This method provides an assessment of the overall methylation status of CpG islands, including some quantitative analysis, but is relatively insensitive and requires large amounts of high molecular weight DNA.

[0010] Another method utilizes bisulfite treatment of DNA to convert all unmethylated cytosines to uracil. The altered DNA is amplified and sequenced to show the methylation status of all CpG sites. However, this method is technically difficult, labor intensive and without cloning amplified products, it is less sensitive than Southern analysis, requiring approximately 10% of the alleles to be methylated for detection.

[0011] Identification of the earliest genetic changes in tumorigenesis is a major focus in molecular cancer research. Diagnostic approaches based on identification of these changes are likely to allow implementation of early detection strategies and novel therapeutic approaches targeting these early changes might lead to more effective cancer treatment.

[0012] About half of all human genes have 5' CpG islands and these islands are usually associated with the 5' regulatory regions of genes (Antequera, et al., Proc. Natl. Acad. Sci. USA 90:11995-11999, 1993). The 5' CpG islands of most nonimprinted genes are thought to remain unmethylated in normal cells but may become methylated during aging or tumorigenesis. Through interactions between methyl CpG binding proteins, histones and histone deacetylase, 5' CpG island methylation can contribute to changes in chromatin that cause transcriptional silencing (Baylin, et al., Adv. Cancer Res. 72:141-196, 1998). Promoter methylation is implicated in the transcriptional silencing of tumor suppressor and mismatch repair genes (e.g. p16, Rb, VHL, hMLH1) in many cancers. Although 13 hypermethylated genes and clones in pancreatic cancers were previously identified (Ueki, et al., Cancer Res. 60:1835-1839, 2000), there almost certainly are others. Costello et al. have estimated that ~400 genes are aberrantly methylated in cancers and have found evidence for tumor-specific pattern of methylation (Costello, et al., Nat. Genet. 24:132-138, 2000). A better description of the pattern of DNA methylation abnormalities in cancer may improve an understanding of the role of DNA methylation in tumorigenesis and identification of differentially methylated CpG islands in cancer may lead to the discovery of novel genes with tumor suppressor properties. Finally, identified genes or loci could be utilized as cancer-specific markers for the early detection of cancer (Belinsky, et al., Proc. Natl. Acad. Sci. USA 95:11891-11896, 1998).

[0013] Pancreatic cancer is the fourth leading cause of cancer death in men and in women and each year ~28,000 Americans die of the disease (Greenlee, et al., CA Cancer J. Clin. 50:7-33, 2000). Frequent genetic changes such as mutational activation of the K-ras oncogene and inactivation of the p16, DPC4, p53, MKK4, STK11, TGFBR2, and TGFBR1 tumor suppressor genes have been described in pancreatic cancer (Goggins, et al., Ann. Oncol. 10:4-8, 1999, Rozenblum, et al., Cancer Res. 57:1731-1734, 1997). Although multiple tumor suppressor pathways have been shown to play a role in pancreatic carcinogenesis, little is known about the contribution of DNA methylation to inactivation of genes in these pathways. Recently, a novel technique, methylated CpG island amplification (MCA), was developed to enrich for methylated CpG rich

sequences. MCA coupled with RDA (MCA/RDA) can recover CpG islands differentially methylated in cancer cells (Toyota, et al., Cancer Res. 59:2307-2312, 1997).

SUMMARY OF THE INVENTION

[0014] The present invention is based on the finding that several genes are newly identified as being differentially methylated in pancreatic cancer. This seminal discovery is useful for cancer screening, risk-assessment, prognosis, minimal-residual disease identification, staging and identification of therapeutic targets. The identification of genes that are methylated in cancer, aging or diseases associated with aging increases the likelihood of finding genes methylated in a particular cancer; increases the sensitivity and specificity of methylation detection; allows methylation profiling using multiple genes; and allows identification of new targets for therapeutic intervention.

[0015] In one aspect of the invention, there is provided a method for detecting pancreatic carcinoma in a subject comprising contacting a nucleic acid-containing specimen from the subject with an agent that provides a determination of the methylation state of at least one gene or associated regulatory region of the gene selected from Tables 1-3 and combinations thereof; and identifying aberrant methylation of regions of the gene or regulatory region, wherein aberrant methylation is identified as being different when compared to the same regions of the gene or associated regulatory region in a subject not having the pancreatic carcinoma, thereby detecting pancreatic carcinoma in the subject. The regions of the gene or regulatory region are contained within CpG rich regions. Illustrative genes are listed in Tables 1-3. In this aspect, the aberrant methylation is hypermethylation when compared to the same regions of the gene or associated regulatory regions in a subject not having pancreatic carcinoma.

[0016] In another aspect of the invention, a pair of primers that hybridize with a target sequence in the gene or associated regulatory region of the gene are utilized to identify genes or regulatory regions associated with pancreatic cancer. In yet another aspect, the nucleic acid-containing specimen includes tissue such as pancreatic ductal

epithelium, pancreatic tissue, stool, blood, or pancreatic fluid. Pancreatic carcinoma includes, but is not limited to, pancreatic ductal adenocarcinoma, chronic pancreatitis, islet cell tumor, or serus cystadenoma.

[0017] In another aspect of the invention, a method of identifying at least one gene silenced by DNA methylation associated with pancreatic carcinoma includes contacting an array of nucleotide sequences representative of a genome with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of silenced genes but not RNA expressed in normal cells corresponding to the cancer cells, under conditions suitable for selective hybridization of nucleic acid subtraction products to complementary nucleotide sequences of the array; and detecting selective hybridization of nucleic acid to a subpopulation of nucleotide sequences of the array, wherein nucleic acid molecules corresponding to RNA expressed in the normal cells corresponding the cancer cells do not hybridize to the subpopulation of nucleotide sequences under the conditions suitable for selective hybridization, whereby the nucleic acid molecules that selectively hybridize to the subpopulation of nucleotide sequences of the array represent epigenetically silenced genes of the cancer cells, thereby identifying at least one epigenetically silenced gene associated with pancreatic cancer.

[0018] Determining the methylation state of the gene includes contacting the nucleic acid-containing specimen with an agent that modifies unmethylated cytosine, amplifying a CpG-containing nucleic acid in the specimen by means of CpG-specific oligonucleotide primers, wherein the oligonucleotide primers distinguish between modified methylated and nonmethylated nucleic acid, and detecting the methylated nucleic acid based on the presence or absence of amplification products produced in the amplifying step. The method includes optionally contacting the amplification products with a methylation sensitive restriction endonuclease. Other methods for determining methylation status of a gene and/or regulatory sequences are well known in the art and are described more fully herein.

[0019] In one aspect, the methylating agent 5 aza 2' deoxycytidine (5Aza-dC) is used to treat cells for further determination of the methylation status. In another aspect, the histone deacetylase inhibitor trichostatin (TSA) is used to treat cells for further determination of the methylation status. In yet another aspect, a combination of 5 aza 2' deoxycytidine (5Aza-dC) and trichostatin (TSA) is utilized. Genes associated with pancreatic carcinoma are listed in Tables 1-3 by way of example. Specifically, such genes include CDH3, reprimo, CLDN5, DR3, FOXE1, LDOC1, LHX1, NEFH, NPTX2, PIG11, SARP2, ST14, SMARCA1, TJP2, UCHL1, WNT7A, or a combination thereof.

[0020] In another aspect of the invention, an amplification primer pair is provided. The amplification primer pair includes a forward primer and a reverse primer as set forth in SEQ ID NOS: 1 to 64, wherein the amplification primer pair amplifies a portion of a gene of Tables 1-3. In one aspect, the amplification primer pair specifically amplifies a methylated 5' regulatory region of the nucleic acid molecule. More specifically, these amplification primer pairs include SEQ ID NOS: 3 and 4, SEQ ID NOS: 7 and 8, SEQ ID NOS: 11 and 12, SEQ ID NOS: 15 and 16, SEQ ID NOS: 19 and 20, SEQ ID NOS: 23 and 24, SEQ ID NOS: 27 and 28, SEQ ID NOS: 31 and 32, SEQ ID NOS: 35 and 36, SEQ ID NOS: 39 and 40, SEQ ID NOS: 43 and 44, SEQ ID NOS: 47 and 48, SEQ ID NOS: 51 and 52, SEQ ID NOS: 55 and 56, SEQ ID NOS: 59 and 60 or SEQ ID NOS: 63 and 64. In another aspect of the invention, the amplification primer pair specifically amplifies a unmethylated 5' regulatory region of the nucleic acid molecule. More specifically, these amplification primer pairs include SEQ ID NOS: 1 and 2, SEQ ID NOS: 5 and 6, SEQ ID NOS: 9 and 10, SEQ ID NOS: 13 and 14, SEQ ID NOS: 17 and 18, SEQ ID NOS: 21 and 22, SEQ ID NOS: 25 and 26, SEQ ID NOS: 29 and 30, SEQ ID NOS: 33 and 34, SEQ ID NOS: 37 and 38, SEQ ID NOS: 41 and 42, SEQ ID NOS: 45 and 46, SEQ ID NOS: 49 and 50, SEQ ID NOS: 53 and 54, SEQ ID NOS: 57 and 58, or SEQ ID NOS: 61 and 62.

[0021] Also included are target sequences to which the amplification primers bind.

[0022] In another aspect, a method for monitoring a therapeutic regimen for treating a subject having pancreatic cancer is provided. The method includes a) obtaining a nucleic acid-containing specimen from the subject prior to therapy; b) contacting the nucleic acid-containing specimen with an agent that provides a determination of the methylation state of at least one gene or associated regulatory region of the gene; c) identifying aberrant methylation of regions of the gene or regulatory region, wherein aberrant methylation is identified as being different when compared to the same regions of the gene or associated regulatory region in a subject not having the pancreatic cancer; and d) determining a change in the methylation state of the gene during therapy, wherein the change is determined by comparing the methylation state of the gene with the methylation state of the same gene from the nucleic acid-containing sample from the same subject prior to therapy, thereby monitoring a therapeutic regimen for treating a pancreatic cancer subject.

[0023] Another aspect of the invention provides a kit containing at least one amplification primer pair including a methylation specific amplification primer pair, an unmethylated specific amplification primer pair, or a combination comprising at least one methylation specific amplification primer pair and at least one unmethylation specific amplification primer pair. The kit further includes a reagent that modifies methylated cytosine residues. In another aspect of the invention, the kit further comprises a methylation sensitive restriction endonuclease. In yet another embodiment, the kit comprises reagents for performing an amplification reaction. Optionally, the kit includes 5-aza-2'-deoxycytidine and/or trichostatin A.

[0024] Another aspect of the invention provides a kit useful for the detection of pancreatic carcinoma in a subject including a carrier means compartmentalized to receive a sample therein; one or more containers comprising a first container containing a reagent which modifies unmethylated cytosine and a second container containing primers for amplification of a CpG-containing nucleic acid, wherein the primers are pairs from SEQ ID NO:1-64. In one aspect of the invention, the kit includes a third container containing a methylation sensitive restriction endonuclease. In this aspect, the modifying reagent is

typically bisulfite. In another illustrative example, the primer hybridizes with a target sequence as set forth in Tables 1-3.

[0025] Another aspect of the invention provides a gene associated with pancreatic carcinoma as listed in Tables 1-3, wherein associated regulatory sequences contain CpG-rich regions. In one aspect, the state of methylation of the CpG-rich regions is determinative of the presence of pancreatic carcinoma in a subject from which the nucleic acid molecule is isolated. In one aspect, hypermethylation of the CpG-rich regions is indicative of the presence of pancreatic carcinoma in a subject from which the nucleic acid is isolated.

BRIEF DESCRIPTION OF THE FIGURES

[0026] **Figure 1a** shows RT-PCR analysis of five genes (*CDH3, NPTX2, SARP2, UCHL1,* and *WNT7A)* in pancreatic cancer cell lines (AsPC1, and MiaPaCa2). Cells were treated with 5Aza-dC alone, TSA alone, or a combination of both and subjected to RNA extraction. Glyceraldehyde-3-phospate dehydrogenase (GAPDH) serves as a RNA control.

[0027] **Figure 1b** shows MSP analysis of five genes (*CDH3, CLDN5, NPTX2, SARP2,* and *TJP2)* in pancreatic cancer cell lines and a nonneoplastic ductal cell line (HPDE). The PCR products in Lances U and M indicated the presence of unmethylated and methylated templates, respectively.

[0028] **Figure 2** is a graphical diagram summarizing the methylation profiles of 11 genes in a panel of 22 pancreatic cancer cell lines determined by MSP. Filled boxes are methylated alleles, open boxes are unmethylated alleles.

[0029] **Figure 3** shows graphical expression patterns of 11 genes aberrantly methylated in pancreatic cancer after treatment with 5Aza-dC, TSA, or a combination of both in four pancreatic cancer cell lines. Cells were treated with 5Aza-dC alone, TSA

alone, or a combination of both, and subjected to oligonucleotide microarray hybridization.

[0030] **Figure 4** shows MSP analysis of three genes (*CLDN5, NPTX2,* and *SARP2)* in a series of normal pancreatic ductal epithelia, primary pancreatic carcinomas, and pancreatic juice samples. The PCR products in Lanes U and M indicated the presence of unmethylated and methylated templates, respectively.

DETAILED DESCRIPTION OF THE INVENTION

[0031] It has been determined that an aberrant methylation state and/or histone deacetylase (HDAC) activity (with the methylation being predominant) of nucleic acids in certain genes, particularly regulatory sequences, is diagnostic for the presence or potential development of a cellular proliferative disorder in subjects bearing the aberrantly methylated nucleic acids. More particularly, the hypermethylation of certain nucleotides localized in CpG islands has been shown to affect the expression of genes associated with the CpG islands; typically such hypermethylated genes have reduced or abolished expression, primarily due to down-regulated transcription. Using a well known technique called methylation specific PCR (MSP), several nucleic acid molecules aberrantly methylated in pancreatic cancer cells were identified.

[0032] The presently disclosed microarray based strategy obviates the disadvantages of previous methods by coupling gene expression status to epigenetic regulation. Furthermore, the approach exploits the observation that global changes in gene expression in cancer genes can be dependent on both dense CpG island methylation and HDAC activity (Cameron et al., Nature Genet. 21:103-107, 1999, which is incorporated herein by reference). The disclosed methods robustly identify genes for which transcriptional repression can have a key role in tumorigenesis. Remarkably, the disclosed genomic screening method allowed an identification of gene hypermethylation events that cluster to specific tumor types, and can simultaneously involve multiple members of a single gene family.

[0033] As used herein, a global change in gene expression refers to a change in any function normally attributed to a cell containing the gene Such global changes in gene expression include, but are not limited to, reactivation of a gene that is epigenetically silenced. The term "epigenetically silenced" or "epigenetic silenced", when used in reference to a gene, means that the gene is not being transcribed, or is being transcribed at a level that is decreased with respect to the level of transcription of the gene in a corresponding control cell (e.g., a normal cell), due to a mechanism other than a genetic change. Epigenetic mechanisms of gene silencing are well known and include, for example, hypermethylation of CpG dinucleotides in a CpG island of the 5' regulatory region of a gene, and structural changes in chromatin due, for example, to histone acetylation, such that gene transcription is reduced or inhibited. Methods for detecting epigenetic silencing of a gene are disclosed herein and include, for example, detecting re-expression (reactivation) of the gene following contact of a cell with an agent that relieves the epigenetic silencing, for example, with a demethylating agent where the silencing is due to hypermethylation.

[0034] Methylated nucleic acid sequences are also provided. As used herein, the term "methylation" or "hypermethylation", when used in reference to a gene, means that cytosine residues of CpG dinucleotides in a CpG island associated with the gene are methylated at the 5'-position, i.e., 5'-methylcytosine. The term "methylation status" is used herein to refer to a relative abundance, including the presence or absence, of methylated cytosine residues of CpG dinucleotides in a CpG island. In general, the cytosine residues in a CpG island are not methylated in a transcriptionally active gene and, therefore, the detection of methylated cytosine residues in a CpG island indicates that expression of the gene is reduced or inhibited. Accordingly, as discussed above, reference herein to a "methylation silenced" gene means that the gene is not being transcribed, or is being transcribed at a level that is decreased with respect to the level of transcription of the gene in a corresponding control cell (generally a normal cell) due to hypermethylation of CpG dinucleotides in a CpG island of the 5' regulatory region of the gene. A consequence of methylation silenced gene expression is that a cell containing

the gene has reduced levels of, or completely lacks, a polypeptide encoded by the gene (i.e., the gene product) such that any function normally attributed to the gene product in the cell is reduced or absent.

[0035] In one embodiment of the invention, a method of identifying an epigenetically silenced gene associated with a cancer can be performed, for example, by contacting an array of nucleotide sequences representative of a genome with nucleic acid subtraction products (i.e., nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of epigenetically silenced genes, but not RNA expressed in normal cells corresponding to the cancer cells) under conditions suitable for selective hybridization of nucleic acid subtraction products to complementary nucleotide sequences of the array; and detecting selective hybridization of nucleic acid subtraction products to a subpopulation of nucleotide sequences of the array, wherein nucleic acid molecules corresponding to RNA expressed in the normal cells corresponding to the cancer cells do not hybridize to the subpopulation of nucleotide sequences under such conditions suitable for selective hybridization, whereby the nucleic acid subtraction products that selectively hybridize to the subpopulation of nucleotide sequences of the array represent epigenetically silenced genes of the cancer cells.

[0036] Reference to "nucleic acid molecules corresponding to RNA" of a cell means RNA such as mRNA or polyA+ RNA, cDNA generated using RNA from the cell as a template, or cRNA generated using RNA or cDNA as a template. For practicing a method of the invention, the nucleic acid molecules corresponding to RNA of a cell generally are detectably labeled, for example, with a radioisotope, a paramagnetic isotope, a luminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, an enzyme, a substrate for an enzyme, a receptor, or a ligand for a receptor; or are capable of being detected, for example, using a detectably labeled probe, such that hybridization of the nucleic acid molecules to nucleotide sequences of the array can be detected.

[0037] As used herein, the term "array of nucleotide sequences representative of a genome" means an organized group of nucleotide sequences that are linked to a solid support, for example, a microchip or a glass slide, wherein the sequences can hybridize specifically and selectively to nucleic acid molecules expressed in a cell. The array is selected based on the organism from which the cells to be examined are derived and/or on a tissue or tissues that are to be examined. Generally, the array is representative of the genome of a eukaryotic cell or cell type, particularly a mammalian cell or cell type, and preferably a human cell, including a cell of one or more tissues, as desired (e.g., pancreatic epithelial cells). In general, an array of probes that is "representative" of a genome will identify at least about 10% of the expressed nucleic acid molecules in a cell, generally at least about 20% or 40%, usually about 50% to 70%, typically at least about 80% or 90%, and particularly 95% to 99% or more of the expressed nucleic acid molecules of a cell or organism. It should be recognized that the greater the representation, the more likely that a method of the invention can identify all genes that are epigenetically silenced in a cancer. Arrays containing nucleotide sequences representative of specified genomes can be prepared using well known methods, or obtained from a commercial source (e.g., Invitrogen Corp.; Affymetrix), as exemplified by a Human Genome U133A chip (Affymetrix, Santa Clara, CA) used in the present studies.

[0038] The agent that reactivates expression of epigenetically silenced genes can be a methyltransferase inhibitor(e.g., 5 aza 2' deoxycytidine; 5Aza-dC), a histone deacetylase inhibitor (e.g., trichostatin A; TSA), or a combination of agents such as a combination of 5Aza-dC and TSA. RNA can be isolated from cells such as cancer cells treated with such an agent or agent, and the RNA, or a cDNA product of the RNA can be contacted with RNA molecules from corresponding cells (e.g., cancer cells) that were not treated with the agent(s) under conditions such that RNA (or cDNA) expressed only in the treated cells can be isolated, thus obtaining nucleic acid subtraction products. Methods for performing a nucleic acid subtraction reaction are well known (Hedrick et al., Nature 308:149-155, 1984, which is incorporated herein by reference), and kits for performing such methods are available from commercial sources (e.g., Gibco/BRL).

[0039] In another embodiment, the methods of the invention identify potential targets for aberrant methylation in pancreatic cancer by analyzing gene expression profiles of cancer cells after exposure to 5Aza-dC and/or TSA. This embodiment includes, in part, a comparison of the methylation status of a gene in a test cell or sample with the methylation status of a corresponding gene in a corresponding cell exhibiting regulated growth. As used herein, the term "corresponding" means a reference material, with which a test material is being compared. Generally, the reference material provides a control or standard with which the test material is compared. For example, reference to a corresponding unmethylated SFRP gene, with respect to an SFRP gene being examined for methylation status, means that the unmethylated SFRP gene is the same type of gene as the a SFRP gene being examined for methylation status, e.g., the test gene and the corresponding unmethylated gene are both human a SFRP1 genes. Reference to a corresponding cell exhibiting regulated growth, with respect to a test cell, generally refers to a normal cell, i.e., a cell that has a cell cycle and growth pattern characteristic of a population of such cells in a healthy individual, for example, a normal pancreatic epithelial cell where the test cell being examined is suspected of being a pancreatic cancer cell.

[0040] A test cell examined according to a method of the invention also can be a primary cell that has been obtained from a subject and placed in culture, for example, for the purpose of establishing a primary cell culture that exhibits substantially the same growth characteristics as the cells from which the culture was established, or for the purpose of treating and/or expanding the cells for readministration to the subject. For example, pancreatic ductal epithelial cells can be obtained from a cancer patient suffering from pancreatic cancer, wherein the cells exhibit methylation silenced expression of one or more genes associated with the cancer. The cells can be treated in culture using one or more agents to be tested for an ability to restore expression of the silenced gene(s), thus providing a means to identify an agent that can be useful for treating the cancer patient, or another patient having a pancreatic cancer characterized by methylation silencing of one or more of the same genes.

[0041] A test cell can be obtained from a subject in any way typically used in clinical setting for obtaining a sample containing the cells. For example, the test cells (or a sample comprising the test cells) can be obtained by a biopsy procedure such as needle biopsy of an organ or tissue containing the cells to be tested. As such, the test cells can be obtained from a gastrointestinal tract sample (e.g., a biopsy of a polyp), a liver sample, a pancreatic tissue sample, a bone marrow sample, a skin sample, a lymph node sample, a kidney sample, a lung sample, a muscle sample, a bone sample, a brain sample, or the like. The test cell also can be a component of a biological fluid, for example, blood, lymph, cerebrospinal fluid, pancreatic juice, saliva, sputum, stool, urine, or ejaculate. If appropriate, the test cells also can be obtained by lavage, for example, for obtaining test cells from the colon, uterus, abdominal cavity, or the like, or using an aspiration procedure, for example, for obtaining a bone marrow sample.

[0042] Where the epigenetic silencing includes methylation silencing, the method for identifying a cell that exhibits or is predisposed to exhibiting unregulated growth is performed by detecting methylation of one or more target genes in the cell. Methylation of a CpG dinucleotide in a CpG island of a gene can be detected using any of various well known methods for detecting CpG methylation of a nucleic acid molecule. Such methods include contacting the gene with one or a series of chemical reagents that selectively modify either unmethylated cytosine residues or methylated cytosine residues, but not both, such that the presence or absence of the modification can be detected; contacting the gene sequence with a methylation sensitive restriction endonuclease, which has a recognition site that includes a CpG dinucleotide, and that cleaves a recognition site either having a methylated cytosine residue of the CpG or lacking a methylated cytosine residue of the CpG, but not both, such that the presence or absence of cleavage of the sequence can be detected; or contacting a nucleic acid molecule comprising the gene with an oligonucleotide probe, primer, or amplification primer pair that selectively hybridizes to the gene sequence and allows a determination to made as to whether the CpG methylation is present. Examples of such methods are provided herein, and modifications and variations on such methods are well known in the art.

[0043] Methylation of a target gene can be detected, for example, by contacting a region comprising a 5' regulatory region of a nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methylated cytosine residue of a CpG dinucleotide, whereby cleavage of the nucleic acid molecule is indicative of methylation and, therefore, methylation silencing of the gene of the test cell. Methylation sensitive restriction endonucleases are well known and include, for example, Acc III, Ban I, BstN I, Msp I, and Xma I. Alternatively, or in addition, methylation silencing can be detected by contacting a region comprising a 5' regulatory region of a nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methylated cytosine residue of a CpG dinucleotide, provided the cytosine residue of the CpG dinucleotide is unmethylated, whereby a lack of cleavage of the nucleic acid molecule is indicative of methylation silencing of the gene of the test cell. Such methylation sensitive restriction endonucleases are exemplified by Acc II, Ava I, BssH II, BstU I, Hpa II, and Not I.

[0044] The presence or absence of cleavage of a nucleic acid molecule comprising a target gene sequence by a methylation sensitive restriction endonuclease can be identified using any method useful for detecting the length or continuity of a polynucleotide sequence. For example, cleavage of the target gene sequence can be detected by Southern blot analysis, which allows mapping of the cleavage site, or using any other electrophoretic method or chromatographic method that separates nucleic acid molecules on the basis of relative size, charge, or a combination thereof. Cleavage of a target gene also can be detected using an oligonucleotide ligation assay, wherein, following contact with the restriction endonuclease, a first oligonucleotide that selectively hybridizes upstream of and adjacent to a restriction endonuclease cleavage site and a second oligonucleotide that selectively hybridizes downstream of and adjacent to the cleavage site are contacted with the target gene sequence, and further contacted with a ligase such that, in the absence of cleavage the oligonucleotides are adjacent to each other and can be ligated together, whereas, in the absence of cleavage, ligation does

not occur. By determining the size or other relevant parameter of the oligonucleotides following the ligation reaction, ligated oligonucleotides can be distinguished from unligated oligonucleotides, thereby providing an indication of restriction endonuclease activity.

[0045] Methylation silencing of a gene also can be detected by contacting a 5' regulatory region of the nucleic acid molecule comprising the gene of the test cell with a chemical reagent that selectively modifies either an unmethylated cytosine residue or a methylated cytosine residue, and detecting a product generated due to the contacting, wherein the product is indicative of methylation of a cytosine residue in a CpG dinucleotide of the gene, thereby detecting methylation silencing of the gene of the test cell. For example, the product can be detected using an electrophoresis method, a chromatography method, a mass spectrometry method, or a combination of such methods.

[0046] In one aspect of the present invention, a nucleic acid molecule comprising the target gene is contacted with a chemical reagent comprising bisulfite ions, for example, sodium bisulfite, which converts unmethylated cytosine residues to bisulfite modified cytosine residues, then the bisulfite ion treated gene sequence is exposed to alkaline conditions, which convert bisulfite modified cytosine residues to uracil residues. Sodium bisulfite reacts readily with the 5,6 double bond of cytosine (but poorly with methylated cytosine) to form a sulfonated cytosine reaction intermediate that is susceptible to deamination, giving rise to a sulfonated uracil. As such, the sulfonate group can be removed by exposure to alkaline conditions, resulting in the formation of uracil. The DNA then can amplified, for example, by PCR, and sequenced to determine the methylation status of all CpG sites. Uracil is recognized as a thymine by Taq polymerase and, upon PCR, the resultant product contains cytosine only at the position where 5 methylcytosine was present in the starting template DNA. By comparing the amount or distribution of uracil residues in the bisulfite ion treated gene sequence of the test cell with a similarly treated corresponding unmethylated gene sequence, detection of a decrease in the amount or distribution of uracil residues in the gene from the test cell is

indicative of methylation of cytosine residues in CpG dinucleotides in the target gene of the test cell. The amount or distribution of uracil residues also can be detected by contacting the bisulfite ion treated target gene sequence, following exposure to alkaline conditions, with an oligonucleotide that selectively hybridizes to a nucleotide sequence of the target gene that either contains uracil residues or that lacks uracil residues, but not both, and detecting selective hybridization (or the absence thereof) of the oligonucleotide.

[0047] As used herein, the term "selective hybridization" or "selectively hybridize" or "specific hybridization" refers to an interaction of two nucleic acid molecules that occurs and is stable under moderately stringent or highly stringent conditions. As such, selective hybridization preferentially occurs, for example, between an oligonucleotide and a target nucleic acid molecule, and not substantially between the oligonucleotide and a nucleic acid molecule other than the target nucleic acid molecule, including not with nucleic acid molecules encoding related but different members of a gene family. Generally, an oligonucleotide useful as a probe or primer that selectively hybridizes to a target nucleic acid molecule is at least about 12 to 15 nucleotides in length, generally at least about 18 to 20 nucleotides in length, usually at least about 21 to 25 nucleotides in length, and particularly about 26 to 35 nucleotides in length or. Examples of oligonucleotides useful in practicing the methods of the invention are disclosed herein in Table 4.

[0048] Conditions that allow for selective hybridization can be determined empirically, or can be estimated based, for example, on the relative GC:AT (or GC:AU) content of the hybridizing oligonucleotide and the target nucleic acid molecule, the length of the hybridizing oligonucleotide, and the number, if any, of mismatches between the oligonucleotide and target sequence to which it is to hybridize (see, for example, Sambrook et al., "Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989)). As such, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the hybridizing nucleic acid molecules. An additional consideration is whether one of the nucleic acids is immobilized, for

example, on a filter. An example of progressively higher stringency conditions is as follows: 2X SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2X SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2X SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1X SSC at about 62°C (high stringency conditions). Hybridization and/or washing can be carried out using only one of these conditions, for example, high stringency conditions, or each of the conditions can be used, for example, for 10 to 15 minutes each, in the order listed above, repeating any or all of the steps listed.

[0049] Selective hybridization of an oligonucleotide with a target gene (e.g., a gene as listed in Tables 1-3) can be detected, for example, by performing the method using an oligonucleotide that includes a detectable label. The detectable label can be any molecule that conveniently can be linked to the oligonucleotide and detected using readily available equipment. For example, the detectable label can be a fluorescent compound such a Cy3, Cy5, Fam, fluorescein, rhodamine, or a green fluorescent protein or enhanced or modified form thereof;, a radionuclide such as sulfur-35, technicium-99, phosphorus-32, tritium or iodine 125; a paramagnetic spin label such as carbon-13, Gd-157, Mn-55, Dy-162, Cr 52, or Fe 56; a luminescent compound such as an aequorin; a chemiluminescent compound; a metal chelate; an enzyme such as luciferase or J-galactosidase, or a substrate for an enzyme; or a receptor or a ligand for a receptor, for example, biotin. The means for detecting the detectable label will be selected based on the characteristics of the label, as will the means for linking the label to an oligonucleotide (see, for example, Hermanson, "Bioconjugate Techniques" (Academic Press 1996), which is incorporated herein by reference).

[0050] Selective hybridization also can be detected, for example, by utilizing the oligonucleotide as a substrate for a primer extension reaction, further contacting the sample with deoxyribonucleotides (dNTPs), including, if desired, a detectable dNTP (e.g., a fluorescently labeled dNTP, a digoxigenin labeled dNTP, or a biotin labeled dNTP), and a DNA dependent DNA polymerase under conditions sufficient for the primer extension reaction to proceed, and detecting a product of the primer extension

reaction. Conditions for performing a primer extension reaction are well known in the art (see, for example, Sambrook et al., supra, 1989).

[0051] The amount or distribution of uracil residues in a bisulfite ion treated nucleic acid molecule comprising a target gene sequence following exposure to alkaline conditions also can be detected using an amplification reaction such as PCR. An amplification reaction is performed under conditions that allow selective hybridization of the forward and reverse primers of an amplification primer pair to the target nucleic acid molecule. Generally, the reaction is performed in a buffered aqueous solution, at about pH 7-9, usually about pH 8. In addition, the reaction generally is performed in a molar excess of primers to target nucleic acid molecule, for example, at a ratio of about 100:1 primer:genomic DNA. Where the amount of the target nucleic acid molecule in a sample is not known, for example, in a diagnostic procedure using a biological sample, a range of primer amounts can be used in samples run in parallel, although generally even the addition of a small amount of primers will result in a sufficient molar excess such that the amplification reaction can proceed.

[0052] The deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and dTTP, can be added to the synthesis mixture either separately or as a mixture, which can further include the primers, in adequate amounts and the resulting solution is heated to about 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the solution is allowed to cool to room temperature, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction, generally a polymerase, and the reaction is allowed to occur under conditions as disclosed herein (see Example 1) or otherwise known in the art. Where the polymerase is heat stable, it can be added together with the other reagents. The polymerase can be any enzyme useful for directing the synthesis of primer extension products, including, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase muteins, reverse transcriptase, and other enzymes, including heat-stable enzymes, as are well known in the art and commercially available. The

amplification products can be identified as methylated or non-methylated by a sequencing method, oligomer restriction (Saiki et al., BioTechnology 3:1008-1012, 1985), allele-specific oligonucleotide probe analysis (Conner et al., Proc. Natl. Acad. Sci. USA 80:278, 1983), oligonucleotide ligation assays (Landegren et al., Science 241:1077, 1988), and the like (see, also, Landegren et al., Science 242:229-237, 1988).

[0053] In one embodiment, a methylation-specific amplification reaction such as methylation-specific PCR (MSP) is used alone, or in combination with bisulfite treatment, to detect the methylation status of a nucleic acid molecule (see U.S. Pat. Nos. 6,265,171; 6,200,756; and 6,017,704, each of which is incorporated herein by reference; see, also, Example 1). MSP is a particularly sensitive method that allows detection of low numbers of methylated alleles and the use of small amounts of a nucleic acid sample, including paraffin-embedded materials, and also can be conveniently adapted to a multiplex analysis, including, for example, simultaneous detection of unmethylated and methylated products in a single sample, thus providing an internal control.

[0054] The amplification primer pairs used in an MSP reaction are designed to specifically distinguish between bisulfite untreated or unmodified DNA, and methylated and unmethylated DNA. MSP primer pairs for unmethylated DNA (unmethylation specific primer pairs) generally have a thymidine residue in the 3' CpG pair to distinguish it from the cytosine residue retained in methylated DNA, and the complement is designed for the antisense primer. MSP primer pairs usually contain relatively few cytosine or guanine residues in the sequence because cytosine is absent in the sense (forward) primer and the guanine is absent in the antisense (reverse) primer; cytosine becomes modified to uracil, which is amplified as thymidine in the amplification product. MSP unmethylation (MSP(U)) specific primer pairs and MSP methylation (MSP(M)) specific are exemplified in Table 4. For example, amplification primer pairs useful for such a method include, for example, methylation specific primer pairs as set forth in SEQ ID NO:3 and 4 for *cadherin3 (CDH3)*; SEQ ID NOS:7 and 8 for *reprimo*; SEQ ID NOS:11 and 12 for *claudin 5 (CLDN5)*; SEQ ID NOS:15 and 16 for *death receptor 3 (DR3)*; SEQ ID NOS:19and 20 for *forkhead box E1 (FOXE1)*; SEQ ID NOS:23 and 24 for *leucine zipper*

down-regulated in cancer 1 (LDOC1); SEQ ID NOS:27 and 28 for *LIM homeobox protein 1 (LHX1)*; SEQ ID NOS:31 and 32 for *neurofilament heavy polypeptide(NEFH)*; SEQ ID NOS:35 and 36 for *neuronal pentraxin II (NPTX2)*; SEQ ID NOS:39 and 40 for *p53-induced protein (PIG11)*; SEQ ID NOS:43 and 44 for *secreted apoptosis related protein 2 (SARP2)*; SEQ ID NOS:47 and 48 for *suppression of tumorigenicity 14 (ST14)*; SEQ ID NOS:51 and 52 for *SWI/SNF-related gene (SMARCA1)*; SEQ ID NOS:55 and 56 for *tight junction protein 2 (TJP2)*; SEQ ID NOS:59 and 60 for *ubiquitin carboxyl-terminal esteraseL1 (UCHL1)*; or SEQ ID NOS:63 and 64 for *wingless-type MMTV integration site family, member 7A (WNT7A)*. Amplification primer pairs useful for such a method include, for example, unmethylation specific primer pairs as set forth in SEQ ID NO:1 and 2 for *cadherin3 (CDH3)*; SEQ ID NOS:5 and 6 for *reprimo*; SEQ ID NOS:9 and 10 for *claudin 5 (CLDN5)*; SEQ ID NOS:13 and 14 for *death receptor 3 (DR3)*; SEQ ID NOS:17 and 18 for *forkhead box E1 (FOXE1)*; SEQ ID NOS:21 and 22 for *leucine zipper down-regulated in cancer 1 (LDOC1)*; SEQ ID NOS:25 and 26 for *LIM homeobox protein 1 (LHX1)*; SEQ ID NOS:29 and 30 for *neurofilament heavy polypeptide(NEFH)*; SEQ ID NOS:33 and 34 for *neuronal pentraxin II (NPTX2)*; SEQ ID NOS:37 and 38 for *p53-induced protein (PIG11)*; SEQ ID NOS:41 and 42 for *secreted apoptosis related protein 2 (SARP2)*; SEQ ID NOS:45 and 46 for *suppression of tumorigenicity 14 (ST14)*; SEQ ID NOS:49 and 50 for *SWI/SNF-related gene (SMARCA1)*; SEQ ID NOS:53 and 54 for *tight junction protein 2 (TJP2)*; SEQ ID NOS:57 and 58 for *ubiquitin carboxyl-terminal esteraseL1 (UCHL1)*; or SEQ ID NOS:61 and 62 for *wingless-type MMTV integration site family, member 7A (WNT7A)*.

[0055] In view of the exemplified methylation-specific and unmethylation-specific primer pairs, and the availability of nucleotide sequences comprising portions of target genes such as those listed in Tables 1-3, it will be recognized that additional methylation-specific and unmethylation-specific primer pairs useful for amplification of a methylated or an unmethylated gene as listed in Tables 1-3 or other identified target genes, as well as for family members related to the listed genes readily can be made.

[0056] Accordingly, in one aspect, MSP is used for detecting the amount or distribution of uracil residues in a bisulfite ion treated target genes following alkaline treatment. Such a method can be performed by contacting the gene sequence with a first amplification primer pair and a second amplification primer pair under conditions suitable for amplification, wherein the first amplification primer pair comprises a forward primer and a reverse primer, and at least one primer of the first primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the target gene that contains uracil residues, and wherein the second amplification primer pair comprises a forward primer and a reverse primer, and both primers of the second primer pair selectively hybridize to a target gene containing cytosine residues, but not to a target gene sequence containing uracil residues, and wherein an amplification product, if any, generated by the first primer pair has a first length, and an amplification product, if any, generated by the second primer pair has a second length, which is different from the first length, whereby the length of the amplification products is indicative of the amount or distribution of uracil residues and, therefore, of methylation of cytosine residues in CpG dinucleotides in the target gene of the test cell.

[0057] The amount or distribution of uracil residues also can be detected by contacting the 5' regulatory region of the gene with a first amplification primer pair and a second amplification primer pair under conditions suitable for amplification, wherein the first amplification primer pair comprises a forward primer and a reverse primer, wherein at least one primer of the first primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and wherein the second amplification primer pair comprises a forward primer and a reverse primer, wherein both primers of the second primer pair selectively hybridize to a nucleotide sequence of the 5' regulatory region of the gene containing cytosine residues, but not to a corresponding nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and wherein an amplification product, if any, generated by the first primer pair has a first length, and wherein an amplification product, if any, generated by the second primer pair has a second length, which is different from the first length, whereby the length of the amplification products is

indicative of uracil residues and, therefore, methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell.

[0058] Methylation silencing of a gene in a cell exhibiting or suspected of exhibiting unregulated growth (e.g., a gene associated with a cancer) also can be identified by contacting a test cell with a demethylating agent, and detecting increased expression of an RNA encoded by the gene as compared to a level of expression of the RNA in a test cell not contacted with a demethylating agent. Such a method can further include detecting methylation, if any, of cytosine residues in a CpG dinucleotide in a CpG island of the 5' regulatory region of the gene in a corresponding cell exhibiting regulated growth, or an extract of the corresponding cell The demethylating agent can be a methyltransferase inhibitor such as DAC. Increased expression of an RNA can be detected using any method for detecting RNA, including, for example, northern blot analysis, a reverse transcription-polymerase chain reaction assay, or selective hybridization to an array of nucleotide sequences as disclosed herein. Accordingly, the methods of the invention can be performed in a high throughput format, wherein the test cell, or extract of the test cell, comprises one of a plurality of test cells, or extracts of the test cells, or a combination thereof; and each of the test cells, or extracts of the test cells, of the plurality is the same or different, or a combination thereof.

[0059] In adapting the methods of the invention to a high throughput format, the test cells, or extracts of the test cell, can be arranged in an array, which can be an addressable array, on a solid support such as a microchip, a glass slide, or a bead, and the cells (or extracts) can be contacted serially or in parallel with an oligonucleotide probe or primer (or primer pair) as disclosed herein. Samples arranged in an array or other reproducible pattern can be assigned an address (i.e., a position on the array), thus facilitating identification of the source of the sample. An additional advantage of arranging the samples in an array, particularly an addressable array, is that an automated system can be used for adding or removing reagents from one or more of the samples at various times, or for adding different reagents to particular samples. In addition to the

convenience of examining multiple samples at the same time, such high throughput assays provide a means for examining duplicate, triplicate, or more aliquots of a single sample, thus increasing the validity of the results obtained, and for examining control samples under the same conditions as the test samples, thus providing an internal standard for comparing results from different assays. Conveniently, cells or extracts at a position in the array can be contacted with two or more oligonucleotide probes or primers (or primer pairs), wherein the oligonucleotides are differentially labeled or comprise a reaction that generates distinguishable products, thus providing a means for performing a multiplex assay. Such assays can allow the examination of one or more, particularly 2, 3, 4, 5, 10, 15, 20, or more genes to identify epigenetically silenced genes in a test cell.

[0060] The present invention also provides oligonucleotides, which can be useful as probes or primers for identifying an epigenetic silenced gene (or the absence thereof). As used herein, the term "oligonucleotide", "polynucleotide", or "nucleic acid molecule" is used broadly to mean a sequence of two or more deoxyribonucleotides or ribonucleotides that are linked together by a phosphodiester bond. An "isolated polynucleotide" is a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, an isolated polynucleotide may include a coding region with its associated regulatory sequences. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. Specifically, methylated forms of nucleotides in a polynucleotide sequence are also included. The term includes single and double forms of DNA.

[0061] The term "gene" also is used herein to refer to a polynucleotide sequence contained in a genome. It should be recognized, however, that a nucleic acid molecule comprising a portion of a gene can be isolated from a cell or can be examined as genomic

DNA, for example, by a hybridization reaction or a PCR reaction. Thus, while in a genome, it may not always be clear as to a specific nucleotide position where a gene begins or ends, for purposes of the present invention, a gene is considered to be a discrete nucleic acid molecule that includes at least the nucleotide sequence set forth in the GenBank Accession Numbers shown in Tables 1-3 for various genes identified and/or examined herein.

[0062] As will be understood by those of skill in the art, when the sequence is RNA, the deoxynucleotides A, G, C, and T of DNA are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the polypeptides.

[0063] The nucleic acid sequence includes the disclosed sequence and sequences that encode conservative variations of the polypeptides encoded by polynucleotides provided herein. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

[0064] Nucleic acid sequences of the invention can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cells" is used.

Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

[0065] In one aspect, the nucleic acid sequences may be inserted into an expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the sequence of interest genetic sequences. Polynucleotide sequence which encode sequence of interest can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the regulatory or expression control sequences. As used herein, the terms "regulatory sequences" and "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The terms "regulatory sequences" and "expression control sequences" are intended to included, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. An example of an expression control sequence includes a promoter.

[0066] A "promoter" is a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3'

regions of the gene. Both constitutive and inducible promoters, are included in the invention (see, e.g., Bitter et al., Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

[0067] In the present invention, the polynucleotide sequences may be inserted into an expression vector which contains a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al., Gene 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem. 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedron promoters).

[0068] Polynucleotide sequences of the invention can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

[0069] "Transformation" means a genetic change induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell). Where the cell is a mammalian cell, the genetic change is generally achieved by introduction of the DNA into the genome of the cell (i.e., stable).

[0070] Thus, a "transformed cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding sequence of interest. Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as E. coli, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired.

[0071] When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the sequence of interest, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

[0072] Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

[0073] In one embodiment, the invention provides substantially purified polypeptides. The term "substantially purified" as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify a polypeptide sequence using standard techniques for protein purification. The substantially pure polypeptide will yield a single major band on a non-reducing polyacrylamide gel. The purity of the polypeptide can also be determined by amino-terminal amino acid sequence analysis.

[0074] Minor modifications of the primary amino acid sequences may result in proteins which have substantially equivalent activity as compared to the unmodified counterpart polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity still exists.

[0075] The polypeptides of the invention also include dominant negative forms of the invention polypeptide which do not have the biological activity of invention polypeptide sequence. A "dominant negative form" of invention is a polypeptide that is structurally similar to the invention polypeptide but does not have wild-type invention function. For example, a dominant-negative invention polypeptide may interfere with wild-type invention function by binding to, or otherwise sequestering, regulating agents, such as upstream or downstream components, that normally interact functionally with the invention polypeptide.

[0076] Due to the clear correlation between methylation of CpG islands and cellular proliferative disorders, in another embodiment of the present invention, there are provided methods for detecting a cellular proliferative disorder in a subject having or at risk for the cellular proliferative disorder. The method includes assaying, in nucleic acid-containing specimen taken from the subject, the methylation state of a gene or its associated regulatory regions, wherein the expression state of the gene or its associated regulatory regions is associated with the presence of the cellular proliferative disorder, and identifying as having a cellular proliferative disorder a subject that has aberrant

methylation of regions of the gene. The method provides for detecting a cellular proliferative disorder in a subject having or at risk for the cellular proliferative disorder by identifying aberrantly methylation of regions of a gene when compared to the same regions of the gene in a subject not having the cellular proliferative disorder.

[0077] The aberrant methylation comprises hypermethylated CpG rich regions (i.e., islands). In one aspect of the present invention, the CpG rich regions are associated with the invention genes, and affect the expression thereof in a methylation state-dependent manner.

[0078] A "cell proliferative disorder" or "cellular proliferative disorder" is any disorder in which the proliferative capabilities of the affected cells is different from the normal proliferative capabilities of unaffected cells. An example of a cell proliferative disorder is neoplasia. Malignant cells (i.e., cancer) develop as a result of a multistep process. Specific, non-limiting examples of cell proliferative disorders associated with increased methylation of CpG-islands are low grade astrocytoma, anaplastic astrocytoma, glioblastoma, medulloblastoma, gastric cancer, colorectal cancer, colorectal adenoma, acute myelogenous leukemia, lung cancer, renal cancer, pancreatic cancer, leukemia, breast cancer, prostate cancer, endometrial cancer and neuroblastoma. The illustrative example of the present invention is pancreatic cancer.

[0079] A cell proliferative disorder as described herein may be a neoplasm. Such neoplasms are either benign or malignant. The term "neoplasm" refers to a new, abnormal growth of cells or a growth of abnormal cells that reproduce faster than normal. A neoplasm creates an unstructured mass (a tumor) which can be either benign or malignant. For example, the neoplasm may be a head, neck, lung, esophageal, stomach, small bowel, colon, bladder, kidney, or cervical neoplasm. The term "benign" refers to a tumor that is noncancerous, e.g. its cells do not proliferate or invade surrounding tissues. The term "malignant" refers to a tumor that is metastastic or no longer under normal cellular growth control.

[0080] A cell proliferative disorder may be an age-associated disorder. Examples of age-associated disorders which are cell proliferative disorders include colon cancer, lung cancer, breast cancer, prostate cancer, and melanoma, amongst others.

[0081] A "nucleic acid containing specimen" includes any type of material containing a nucleic acid to be subject to invention methods. The nucleic acid may be contained in a biological sample. Such samples include but are not limited to any bodily fluid, such as a serum, urine, saliva, blood, cerebrospinal fluid, pleural fluid, ascites fluid, sputum, stool, or a biopsy sample.

[0082] Samples or specimens include any CpG-rich DNA sequence, whatever the origin, as long as the sequence is detectably present in a sample. While routine diagnostic tests may not be able to identify cancer cells in these samples, the method of the present invention identifies neoplastic cells derived from the primary tumor or from a metastases. The method includes non-invasive sampling (e.g., bodily fluid) as well as invasive sampling (e.g., biopsy). The sample of DNA of the subject may be serum, plasma, lymphocytes, urine, sputum, bile, stool, cervical tissue, saliva, tears, pancreatic juice, duodenal juice, cerebral spinal fluid, regional lymph node, histopathologic margins, and any bodily fluid that drains a body cavity or organ. Therefore, the method provides for the non-invasive detection of various tumor types including head and neck cancer, lung cancer, esophageal cancer, stomach cancer, small bowel cancer, colon cancer, bladder cancer, kidney cancers, pancreatic cancers, cervical cancer and any other organ type that has a draining fluid accessible to analysis. For example, neoplasia of regional lymph nodes associated with a primary mammary tumor can be detected using the method of the invention. Regional lymph nodes for head and neck carcinomas include cervical lymph nodes, prelaryngeal lymph nodes, pulmonary juxta-esophageal lymph nodes and submandibular lymph nodes. Regional lymph nodes for mammary tissue carcinomas include the axillary and intercostal nodes. Samples also include urine DNA for bladder cancer or plasma or saliva DNA for head and neck cancer patients.

[0083] Any nucleic acid sample, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids in accordance with the present invention, provided it contains, or is suspected of containing, a nucleic acid sequence containing a target locus (e.g., CpG-containing nucleic acid). In general, the CpG-containing nucleic acid is DNA. However, invention methods may employ, for example, samples that contain DNA, or DNA and RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded, or a DNA-RNA hybrid may be included in the sample. A mixture of nucleic acids may also be employed. The specific nucleic acid sequence to be detected may be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be studied be present initially in a pure form; the nucleic acid may be a minor fraction of a complex mixture, such as contained in whole human DNA. The nucleic acid-containing sample used for detection of methylated CpG may be from any source including, but not limited to, brain, colon, urogenital, lung, renal, pancreas, liver, esophagus, stomach, hematopoietic, breast, thymus, testis, ovarian, and uterine tissue, and may be extracted by a variety of techniques such as that described by Maniatis, et al. (Molecular Cloning: a Laboratory Manual, Cold Spring Harbor, NY, pp 280, 281, 1982).

[0084] The nucleic acid of interest can be any nucleic acid where it is desirable to detect the presence of a differentially methylated CpG island. The CpG island comprises a CpG island located in a gene or regulatory region for a gene. A "CpG island" is a CpG rich region of a nucleic acid sequence. The nucleic acid sequence may include, for example, MICP 1-42. However, any gene or nucleic acid sequence of interest containing a CpG sequence can provide diagnostic information (i.e., via its methylation state) using invention methods.

[0085] Moreover, these markers can also be multiplexed in a single amplification reaction to generate a low cost, reliable cancer screening test for many cancers simultaneously.

[0086] A combination of DNA markers for CpG-rich regions of nucleic acid may be amplified in a single amplification reaction. The markers are multiplexed in a single amplification reaction, for example, by combining primers for more than one locus. For example, DNA from a urine sample can be amplified with three different randomly labeled primer sets, such as those used for the amplification of the MICP38-42 loci, in the same amplification reaction. The reaction products are separated on a denaturing polyacrylamide gel, for example, and then exposed to film for visualization and analysis. By analyzing a panel of markers, there is a greater probability of producing a more useful methylation profile for a subject.

[0087] If the sample is impure (e.g., plasma, serum, stool, ejaculate, sputum, saliva, cerebrospinal fluid, or blood or a sample embedded in paraffin), it may be treated before amplification with a reagent effective for lysing the cells contained in the fluids, tissues, or animal cell membranes of the sample, and for exposing the nucleic acid(s) contained therein. Methods for purifying or partially purifying nucleic acid from a sample are well known in the art (e.g., Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, 1989, herein incorporated by reference).

[0088] In order to detect a differential methylation state for a gene or CpG-containing region of interest, invention methods include any means known in the art for detecting such differential methylation. For example, detecting the differential methylation may include contacting the nucleic acid-containing specimen with an agent that modifies unmethylated cytosine, amplifying a CpG-containing nucleic acid in the specimen by means of CpG-specific oligonucleotide primers, wherein the oligonucleotide primers distinguish between modified methylated and nonmethylated nucleic acid, and detecting the methylated nucleic acid based on the presence or absence of amplification products produced in the amplifying step. This embodiment includes the PCR-based methods described in U.S. Patent No. 5,786,146, incorporated herein in its entirety.

[0089] For the first time, the methylation state of a number of genes has been correlated with cell proliferative disorders, and more specifically pancreatic cancers. Examples of such genes and their NCBI accession numbers, including the location of the clone, are set out in Tables 1-3.

[0090] In another embodiment, detection of differential methylation is accomplished by contacting a nucleic acid sample suspected of comprising a CpG-containing nucleic acid with a methylation sensitive restriction endonuclease that cleaves only unmethylated CpG sites under conditions and for a time to allow cleavage of unmethylated nucleic acid. The sample is further contacted with an isoschizomer of the methylation sensitive restriction endonuclease, that cleaves both methylated and unmethylated CpG-sites, under conditions and for a time to allow cleavage of methylated nucleic acid. Oligonucleotides are added to the nucleic acid sample under conditions and for a time to allow ligation of the oligonucleotides to nucleic acid cleaved by the restriction endonuclease, and the digested nucleic acid is amplified by conventional methods such as PCR wherein primers complementary to the oligonucleotides are employed. Following identification, the methylated CpG-containing nucleic acid can be cloned, using method well known to one of skill in the art (see Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, 1989).

[0091] As used herein, a "methylation sensitive restriction endonuclease" is a restriction endonuclease that includes CG as part of its recognition site and has altered activity when the C is methylated as compared to when the C is not methylated. Preferably, the methylation sensitive restriction endonuclease has inhibited activity when the C is methylated (e.g., SmaI). Specific non-limiting examples of a methylation sensitive restriction endonucleases include Sma I, BssHII, or HpaII. Such enzymes can be used alone or in combination. Other methylation sensitive restriction endonucleases will be known to those of skill in the art and include, but are not limited to SacII, EagI, and BstUI, for example. An "isoschizomer" of a methylation sensitive restriction endonuclease is a restriction endonuclease which recognizes the same recognition site as a methylation sensitive restriction endonuclease but which cleaves both methylated and

unmethylated CGs. One of skill in the art can readily determine appropriate conditions for a restriction endonuclease to cleave a nucleic acid (see Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, 1989). Without being bound by theory, actively transcribed genes generally contain fewer methylated CGs than in other genes.

[0092] In one embodiment of the invention, a nucleic acid of interest is cleaved with a methylation sensitive endonuclease. In one aspect, cleavage with the methylation sensitive endonuclease creates a sufficient overhang on the nucleic acid of interest. Following cleavage with the isoschizomer, the cleavage product can still have a sufficient overhang. An "overhang" refers to nucleic acid having two strands wherein the strands end in such a manner that a few bases of one strand are not base paired to the other strand. A "sufficient overhang" refers to an overhang of sufficient length to allow specific hybridization of an oligonucleotide of interest. In one embodiment, a sufficient overhang is at least two bases in length. In another embodiment, the sufficient overhang is four or more bases in length. An overhang of a specific sequence on the nucleic acid of interest may be desired in order for an oligonucleotide of interest to hybridize. In this case, the isoschizomer can be used to create the overhang having the desired sequence on the nucleic acid of interest.

[0093] In another aspect of this embodiment, the cleavage with a methylation sensitive endonuclease results in a reaction product of the nucleic acid of interest that has a blunt end or an insufficient overhang. In this embodiment, an isoschizomer of the methylation sensitive restriction endonuclease can create a sufficient overhang on the nucleic acid of interest. "Blunt ends" refers to a flush ending of two stands, the sense stand and the antisense strand, of a nucleic acid.

[0094] Once a sufficient overhang is created on the nucleic acid of interest, an oligonucleotide is ligated to the nucleic acid cleaved of interest which has been cleaved by the methylation specific restriction endonuclease. "Ligation" is the attachment of two nucleic acid sequences by base pairing of substantially complementary sequences and/or

by the formation of covalent bonds between two nucleic acid sequences. In one aspect of the present invention, an "oligonucleotide" is a nucleic acid sequence of about 2 up to about 40 bases in length. It is presently preferred that the oligonucleotide is from about 15 to 35 bases in length.

[0095] In one embodiment, an adaptor is utilized to create DNA ends of desired sequence and overhang. An "adaptor" is a double-stranded nucleic acid sequence with one end that has a sufficient single-stranded overhang at one or both ends such that the adaptor can be ligated by base-pairing to a sufficient overhang on a nucleic acid of interest that has been cleaved by a methylation sensitive restriction enzyme or an isoschizomer of a methylation sensitive restriction enzyme. Adaptors can be obtained commercially, or two oligonucleotides can be utilized to form an adaptor. Thus, in one embodiment, two oligonucleotides are used to form an adaptor; these oligonucleotides are substantially complementary over their entire sequence except for the region(s) at the 5' and/or 3' ends that will form a single stranded overhang. The single stranded overhang is complementary to an overhang on the nucleic acid cleaved by a methylation sensitive restriction enzyme or an isoschizomer of a methylation sensitive restriction enzyme, such that the overhang on the nucleic acid of interest will base pair with the 3' or 5' single stranded end of the adaptor under appropriate conditions. The conditions will vary depending on the sequence composition (GC vs AT), the length, and the type of nucleic acid (see Sambrook et al., Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY, 1998).

[0096] Following the ligation of the oligonucleotide, the nucleic acid of interest is amplified using a primer complementary to the oligonucleotide. Specifically, the term "primer" as used herein refers to a sequence comprising two or more deoxyribo-nucleotides or ribonucleotides, preferably more than three, and more preferably more than eight, wherein the sequence is capable of initiating synthesis of a primer extension product, which is substantially complementary to a nucleic acid such as an adaptor or a ligated oligonucleotide. Environmental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA

polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. In one embodiment, the primer is an oligodeoxyribo-nucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 12-20 or more nucleotides, although it may contain fewer nucleotides.

[0097] Primers of the invention are designed to be "substantially" complementary to each strand of the oligonucleotide to be amplified and include the appropriate G or C nucleotides as discussed above. This means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to perform. In other words, the primers should have sufficient complementarity with a 5' and 3' oligonucleotide to hybridize therewith and permit amplification of CpG containing nucleic acid sequence.

[0098] Primers of the invention are employed in the amplification process which is an enzymatic chain reaction that produces exponential quantities of target locus relative to the number of reaction steps involved (e.g., polymerase chain reaction or PCR). Typically, one primer is complementary to the negative (-) strand of the locus and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA Polymerase I (Klenow) and nucleotides, results in newly synthesized + and - strands containing the target locus sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (i.e., the target locus sequence) defined by the primer. The product of the chain reaction is a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

[0099] The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al. (Tetrahedron Letters, 22:1859-1862, 1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

[0100] Where the CpG-containing nucleic acid sequence of interest contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as a template for the amplification process. Strand separation can be effected either as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished using various suitable denaturing conditions, including physical, chemical, or enzymatic means, the word "denaturing" includes all such means. One physical method of separating nucleic acid strands involves heating the nucleic acid until it is denatured. Typical heat denaturation may involve temperatures ranging from about 80° to 105°C for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or by the enzyme RecA, which has helicase activity, and in the presence of riboATP, is known to denature DNA. The reaction conditions suitable for strand separation of nucleic acids with helicases are described by Kuhn Hoffmann-Berling (CSH-Quantitative Biology, 43:63, 1978) and techniques for using RecA are reviewed in C. Radding (Ann. Rev. Genetics, 16:405-437, 1982).

[0101] When complementary strands of nucleic acid or acids are separated, regardless of whether the nucleic acid was originally double or single stranded, the separated strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis is performed under conditions allowing hybridization of primers to templates to occur. Generally synthesis occurs in a buffered aqueous solution, generally at a pH of about 7-9. Preferably, a molar excess (for genomic nucleic acid, usually about 108:1 primer:template) of the two oligonucleotide primers is added to the

buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process of the invention is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. a large molar excess is preferred to improve the efficiency of the process.

[0102] The deoxyribonucleoside triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the solution is allowed to cool to approximately room temperature, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction (called herein "agent for polymerization"), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization may also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction may occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40°C. Most conveniently the reaction occurs at room temperature.

[0103] The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase muteins, reverse transcriptase, and other enzymes, including heat-stable enzymes (i.e., those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation such as Taq DNA polymerase, and the like). Suitable enzymes will facilitate combination of the

nucleotides in the proper manner to form the primer extension products which are complementary to each locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents for polymerization, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

[0104] Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. However, alternative methods of amplification have been described and can also be employed. PCR techniques and many variations of PCR are known. Basic PCR techniques are described by Saiki et al. (1988 Science 239:487-491) and by U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159, which are incorporated herein by reference.

[0105] The conditions generally required for PCR include temperature, salt, cation, pH and related conditions needed for efficient copying of the master-cut fragment. PCR conditions include repeated cycles of heat denaturation (i.e. heating to at least about 95°C.) and incubation at a temperature permitting primer: adaptor hybridization and copying of the master-cut DNA fragment by the amplification enzyme. Heat stable amplification enzymes like the pwo, Thermus aquaticus or Thermococcus litoralis DNA polymerases are commercially available which eliminate the need to add enzyme after each denaturation cycle. The salt, cation, pH and related factors needed for enzymatic amplification activity are available from commercial manufacturers of amplification enzymes.

[0106] As provided herein an amplification enzyme is any enzyme which can be used for in vitro nucleic acid amplification, e.g. by the above-described procedures. Such amplification enzymes include pwo, Escherichia coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, Thermus aquaticus (Taq) DNA polymerase, Thermococcus litoralis DNA polymerase, SP6 RNA polymerase, T7 RNA polymerase, T3 RNA polymerase, T4 polynucleotide

kinase, Avian Myeloblastosis Virus reverse transcriptase, Moloney Murine Leukemia Virus reverse transcriptase, T4 DNA ligase, E. coli DNA ligase or Q.beta. replicase. Preferred amplification enzymes are the pwo and Taq polymerases. The pwo enzyme is especially preferred because of its fidelity in replicating DNA.

[0107] Once amplified, the nucleic acid can be attached to a solid support, such as a membrane, and can be hybridized with any probe of interest, to detect any nucleic acid sequence. Several membranes are known to one of skill in the art for the adhesion of nucleic acid sequences. Specific non-limiting examples of these membranes include nitrocellulose (NITROPURE) or other membranes used in for detection of gene expression such as polyvinylchloride, diazotized paper and other commercially available membranes such as GENESCREEN, ZETAPROBE (Biorad), and NYTRAN Methods for attaching nucleic acids to these membranes are well known to one of skill in the art. Alternatively, screening can be done in a liquid phase.

[0108] In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

[0109] An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, e.g., high stringency conditions, or each of the conditions can be used, e.g., for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular

hybridization reaction involved, and can be determined empirically. In general, conditions of high stringency are used for the hybridization of the probe of interest.

[0110] The probe of interest can be detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator, or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation.

[0111] In one embodiment, representational difference analysis (RDA, see Lisitsyn et al., Science 259:946-951, 1993, herein incorporated by reference) can be performed on CpG-containing nucleic acid following MCA. MCA utilizes kinetic and subtractive enrichment to purify restriction endonuclease fragments present in one population of nucleic acid fragments but not in another. Thus, RDA enables the identification of small differences between the sequences of two nucleic acid populations. RDA uses nucleic acid from one population as a "tester" and nucleic acid from a second population as a "driver" in order to clone probes for single copy sequences present in (or absent from) one of the two populations. In one embodiment, nucleic acid from a "normal" individual or sample, not having a disorder such as a cell-proliferative disorder is used as a "driver," and nucleic acid from an "affected" individual or sample, having the disorder such as a cell proliferative disorder is used as a "tester." In one embodiment, the nucleic acid used as a "tester" is isolated from an individual having a cell proliferative disorder such as low grade astrocytoma, anaplastic astrocytoma, glioblastoma, medulloblastoma, gastric cancer, colorectal cancer, colorectal adenoma, acute myelogenous leukemia, leukemia, lung cancer, renal cancer, breast cancer, prostate cancer, endometrial cancer and neuroblastoma. The nucleic acid used as a "driver" is thus normal astrocytes, normal glial cells, normal brain cells, normal gastric cells, normal colorectal cells, normal leukocytes, normal lung cells, normal kidney cells, normal breast cells, normal prostate cells, normal uterine cells, and normal neurons, respectively. In an additional embodiment, the nucleic acid used as a "driver" is isolated from an individual having a cell proliferative disorder such as low grade astrocytoma, anaplastic

astrocytoma, glioblastoma, medulloblastoma, gastric cancer, colorectal cancer, colorectal adenoma, acute myelogenous leukemia, leukemia, lung cancer, renal cancer, breast cancer, prostate cancer, endometrial cancer and neuroblastoma. The nucleic acid used as a "tester" is thus normal astrocytes, normal glial cells, normal brain cells, normal gastric cells, normal colorectal cells, normal leukocytes, normal lung cells, normal kidney cells, normal breast cells, normal prostate cells, normal uterine cells, and normal neurons, respectively. One of skill in the art will readily be able to identify the "tester" nucleic acid useful with to identify methylated nucleic acid sequences in given "driver" population.

[0112] The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Therefore, in accordance with another embodiment of the present invention, there is provided a kit it useful for the detection of a cellular proliferative disorder in a subject having or at risk for the cellular proliferative disorder. Invention kits include a carrier means compartmentalized to receive a sample in close confinement therein, one or more containers comprising a first container containing a reagent which modifies unmethylated cytosine and a second container containing primers for amplification of a CpG-containing nucleic acid, wherein the primers distinguish between modified methylated and nonmethylated nucleic acid, and optionally, a third container containing a methylation sensitive restriction endonuclease. Primers contemplated for use in accordance with the invention include those that would amplify sequences or fragments thereof as set forth in SEQ ID NOS: 1-64.

[0113] In another aspect, the kit is ideally suited for high throughput automated analysis. Thus, the kit further comprises a plurality of nucleotides arranged in an array on a solid support such as a microchip, a glass slide, or a bead, which can be contacted serially or in parallel with test samples as prepared above.

[0114] Carrier means are suited for containing one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. In view of the description provided herein of

invention methods, those of skill in the art can readily determine the apportionment of the necessary reagents among the container means. For example, one of the container means can comprise a container containing an oligonucleotide for ligation to nucleic acid cleaved by a methylation sensitive restriction endonuclease. One or more container means can also be included comprising a primer complementary to the oligonucleotide. In addition, one or more container means can also be included which comprise a methylation sensitive restriction endonuclease. One or more container means can also be included containing an isoschizomer of the methylation sensitive restriction enzyme.

[0115] It should be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the restriction enzyme" includes reference to one or more restriction enzymes and equivalents thereof known to those skilled in the art, and so forth.

[0116] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[0117] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies which are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

[0118] The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLE 1

Materials and Methods

A. Collection and Preparation of Pancreatic Cell Lines

[0119] Twenty-two human pancreatic cancer cell lines and immortal cell line derived from normal human pancreatic ductal epithelium (HPDE; provided by Dr. Ming-Sound Tsao, University of Toronto, Toronto, Ontario, Canada) were used in this study. Primary pancreatic carcinoma tissues were obtained from surgical specimens resected at The Johns Hopkins Medical Institutions and microdissected to enrich neoplastic cellulairity as described in Ueki, *Cancer Res.*, 60:1835-1839,2000; Ueki, *Cancer Res.*, 61:8540-8546, 2001). Normal pancreatic duct epithelial cells were selectively microdissected from resected pancreata from 10 patients (mean age, 64.3 years; range, 36-83 years) with various pancreatic disorders using a laser capture microdissection system. Pancreatic juice samples were collected from 37 patients (mean age, 62.9 years; range 31-81 years) undergoing pancreaticduodenectormy for pancreatic ductal adenocarcinoma (24 patients), chronic pancreatitis (8 patients), islet cell tumor (4 patients), and serous cystadenoma (1 patient). Pancreatic juice was retrieved by direct aspiration from the transected pancreatic duct at the time of surgical resection.

B. Treatment with 5Aza-dC and/or TSA

[0120] Four pancreatic cancer cell lines (AsPC1, Hs766T, MiaPaCa2, and Panc1) were treated with 5Aza-dC (sigma, St. Louis, MO) and TSA (Sigma), wither alone or in combination. Cells were exposed continuously to 5Aza-dC (1 μM) for 4 days or to TSA (1 μM) for 24h. It was observed that treatment of the cell lines with 5Aza-dC (1 μM) for 4 days resulted in marked induction of several genes silenced by aberrant methylation without evidence for cell death. Mock-treated cells were cultured with the equivalent

volume of PBS alone. For combined treatment, these cells were cultured in the presence of 5Aza-dC (1 μM) for 3 days and then treated for another 24h with TSA (0.5 μM).

C. Oligonucleotide Array Hybridization

[0121] Total RNA was isolated from cultured cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy Mini Kit (Qiagen, Valencia, CA). First- and second-stranded cDNA was synthesized from 10 μg of total RNA using T7-dT) primer (Genset Corp., La Jolla, CA) and SuperScript Choice system (Invitrogen). Labeled cRNA was synthesized from the purified cDNA by *in vitro* transcription reaction using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY) at 37°C for 6h. The cRNA was fragmented at 94°C for 35 min. in a fragmentation buffer [40 mM Tris-acetate @ pH 8.1, 100 mM potassium acestate, and 30 mM magnesium acetate]. The fragmented cRNA was then hybridized to the Human Genome U133A chips (Affymetrix, Snata Clara, CA) with 18462 unique gene/EST transcripts at 45°C for 16h. The washing and staining procedure was performed in the Affymetrix Fluidics Station according to the manufacturer's instructions. The probes were then scanned using a laser scanner, and signal intensity for each transcript (background-subtracted and adjusted for noise) was calculated using Microarray Suite Software 5.0 (Affymetrix).

D. Reverse Transcription-PCR (RT-PCR)

[0122] Four μg of total RNA were reverse transcribed using Superscript II (Invitrogen). PCR reaction was performed under the following conditions: (a) 95°C for 5 min.; (b) 30-35 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s; and (c) a final extension of 4 min. at 72°C. Primer sequences were
5'-CTCTGTTTAGCACTGATAATG-3' (forward), SEQ ID NO:65 and
5'-TTTATTAGACTTGAGCTGATTC-3' (reverse), SEQ ID NO:66 for *CDH3*;
5'-CATCGAGCTGCTCATCAAC-3' (forward), SEQ ID NO:67 and
5'-CTGCTCTTGTCCAAGGATC-3' (reverse), SEQ ID NO:68 for *CNPTX2*;
5'-CTGGCCCGAGATGCTTAAG-3' (forward), SEQ ID NO:69 and
5'-TATTTTCATCCTCAGTGCAAAC-3' (reverse), SEQ ID NO:70 for *SARP2*;

5'-CTTCATGAAGCAGACCATTG-3' (forward), SEQ ID NO:71 and 5'-ATCATGGGCTGCCTGTATG-3' (reverse), SEQ ID NO:72 for *UCHL1*; and 5'-CGGGAGATCAAGCAGAATC-3' (forward), SEQ ID NO:73 and 5'-AACGGCCTCGTTGTACTTG-3' (reverse), SEQ ID NO:74 for *WNT7a*. Glyceraldehyde-2-phosphate dehydrogenase (*GAPDH*) was also amplified as a control to ensure the cDNA integrity.

E. Bisulfite Treatment and Methylation-Specific PCR (MSP)

[0123] Methylation status of the 5' CpG island of each gene was determined by MSP as described by Herman, *Proc. Natl. Acad. Sci. USA*, 93:9821-9826, 1996. DNA samples were treated with sodium bisulfite (Sigma) for 16 h at 50°C. After purification with the Wizard DNA clean-up system (Promega, Madison, WI), 1 μg of bisulfite-treated DNA was amplified using primers specific for either methylated or unmethylated DNA. Primers were designed to detect the sequence differences between methylated and unmethylated DNA as a result of bisulfite modification, and each primer pair contained at least four CpG sites to provide optimal specificity. Primer sequences for 16 genes analyzed in this study are disclosed in Table 4. PCR conditions were as follows: (a) 95°C for 5 min.; (b) 40 cycles of 95°C for 20 s, 60°C-62°C for 20 s, and 72°C for 30 s; and (c) a final extension of 4 min. at 72°C. 5 μl of each PCR product were loaded onto 3% agarose gels and visualized by ethidium bromide staining.

F. Statistics

[0124] Fold change analysis of signal intensities obtained from oligonucleotide microarrays between the two treatment groups was performed using Data Mining software (Affymetrix). The frequency of aberrant methylation in pancreatic juice samples between patients with pancreatic cancer and those with other pancreatic diseases was compared using Fisher's exact probability test.

EXAMPLE 2

Identification of Genes Induced by 5Aza-dC in Pancreatic Cancer Cell Lines

[0125] The global changes in gene expression profiles induced by 5Aza-dC in four pancreatic cancer cell lines (AsPC1, Hs766T, MiaPaCa2, and Panc1) were determined using the Affymetrix U133 oligonucleotide microarrays with 18462 probes sets (transcripts) covering over 13000 full-length sequences of known genes.. Compared with mock-treated counterparts, 5Aza-dC treatment resulted in a substantial increase (> 5-fold) in signal intensities of 225 transcripts (1.2% of the 18462 trancripts analyzed) in AsPC1, 167 transcripts (0.9%) in Hs766T, 251 transcripts (1.4%) in MiaPaCa2, and 116 transcripts (0.6%) in Panc1. The gene expression changes in nonneoplastic ductal cell line HPDE treated with 5Aza-dC identified 101 transcripts (0.5%) whose expression was induced after drug treatment. Forty-one transcripts that were also reactivated in the nonneoplastic HPDE cell line were excluded from the 631 candidates to identify genes that were aberrantly methylated specifically in pancreatic cancer but not in normal pancreatic ductal epithelium. This left 590 transcripts (487 known genes and 103 expressed sequence tags) specifically up-regulated by 5Aza-dC treatment in pancreatic cancers. Of these 487 known genes, 10 were represented by two or more probes sets, resulting in 475 genes identified as markedly up-regulated by 5Aza-dC treatment in one or more pancreatic cancer cell lines but not in the nonneoplastic HPDE cells. Table 1 lists the data for the 50 most interesting genes identified..

[0126] Of note, this large panel of genes includes several cancer testis antigens (G antigens, and so forth), IFN-related genes (IFN-stimulated gene, and so forth), and imprinted genes (insulin-like growth factor II), which is consistent with previous reports of genes inducible by 5Aza-dC. Although several genes have been reported to be induced by 5Aza-dC treatment independent of the methylation status of their 5' CpG islands, many of the genes identified here may represent potential targets for aberrant methylation in pancreatic cancer. In fact, our approach identified several genes previously reported to be aberrantly methylated in pancreatic and other cancers, such as *CACNA1G, CDKN1A/p21, p57KIP2, stratifin/14-3-3σ, and TIMP-3.*

EXAMPLE 3

Identification of Genes Induced by TSA in Pancreatic Cancer Cell Lines

[0127] The global changes in gene expression profiles induced by the histone deacetylase inhibitor TSA in the same four pancreatic cancer cell lines were analyzed. Treatment with TSA resulted in a marked induction of 424 transcripts (2.3% of the 18642 transcripts analyzed) in AsPC1, 349 transcripts (1.9%) in Hs766T, 207 transcripts (1.1%) in MiaPaCa2, and 459 transcripts (2.5%) in Panc1. Overall, 1196 transcripts (6.5%) including 965 genes and 231 expressed sequence tags were induced 5-fold or greater by TSA in one or more of the four pancreatic cancer cell lines. These include a large panel of novel targets for silencing by histone deacetylation including several known tumor suppressor genes or cell cycle-regulatory genes (*ING1, p57KIP2, CHES1, CHFR, GADD45B,* and others). Many of the genes induced by TSA treatment were also induced by 5Aza-dC treatment alone, suggesting a role for both DNA methylation and histone deacetylation in the transcriptional regulation of these genes. Interestingly, treatment of Hs766T with TSA but not with 5Aza-dC resulted in a significant increase in expression of many cancer testis antigens (e.g., G antigens), whereas these genes were inducible by treatment with 5Aza-dC but not with TSA in the other three cell lines.

EXAMPLE 4

Identification of Genes Induced by Treatment of Pancreatic Cancer Cell Lines with Both Agents

[0128] The gene expression profiles in four pancreatic cancer cell lines after combined treatments with 5 Aza-dC and TSA was determined. Treatment with both agents resulted in induction of 422 (2.3%) of the 18462 transcripts in AsPC1, 304 transcripts (1.6%) in Hs766T, 243 transcripts (1.3%) in MiaPaCa2, and 196 transcripts (1.1%) in Panc1. Overall, 857 transcripts (1.6%) corresponding to 707 genes and 150 expressed sequence tags were induced in at least one of the four pancreatic cancer cell lines. The 707 genes induced by the combined treatment include several genes known to

be aberrantly methylated in cancers (e.g., *p16* and *MLH1*), supporting a previous notion that some of the genes with densely methylated CpG islands are reexpressed by a combined treatment with 5Aza-dC and TSA. Although treatment of all of the four pancreatic cancer cell lines with 5Aza-dC alone or TSA alone did not result in apparent changes in their phenotypes during the treatment period, combined treatment of certain pancreatic cancer cell lines with 5Aza-dC and TSA induced cell death in a small fraction of cells.

EXAMPLE 5

Expression and Methylation Analysis of Selected Genes in Pancreatic Cancer Cell Lines

[0129] Novel targets for aberrant methylation in pancreatic cancer were identified using 16 candidate genes that have been reported to be cancer associated or considered functionally important from the list of 457 genes identified as markedly (> 5-fold) up-regulated by 5Aza-dC treatment in one or more of pancreatic cancer cell lines but not in the nonneoplastic HPDE cells. The genes are listed in Table 4. A literature search using PubMed revealed that 14 of the 16 genes have not been implicated for aberrant methylation in any tumor type, whereas *SARP2* (also termed (*SFRP1*) and *TJP2* (alos termed *ZO-2*) have been recently reported to be frequently methylated in colorectal and pancreatic cancers, respectfully. All of the 16 genes were identified as having CpC-rick sequences fulfilling the criteria of CpG island [GC content > 50%, CpG:GpC ratio > 0.6, and minimum length (200 bp)] in their 5' regions.

[0130] RT-PCR was performed on 5 (*CDH3, NPTX2, SARP2, UCHL1,* and *WNT7A*) of these 16 genes in two pancreatic cancer cell lines (AsPC1 and MiaPaCa2) to compare the results with the corresponding microarray data and found concordant results (Figure 1A).

[0131] The methylation status of the 16 genes was then determined in the nonneoplastic HPDE cells. By MSP, five of these genes (*DR3, LDOC1, NEFH, PIG11,*

and *SMARCA1*) showed partial methylation in HPDE and were excluded from further analysis. The remaining 11 genes were completely unmethylated in HPDE, and the methylation status of these 11 genes was determined in a panel of 22 pancreatic cancer cell lines. Hypermethylation of all 11 of these genes was found in varying frequencies as depicted in Figure 1B. The most frequently methylated was *UCHL1* (methylated in 100%), followed by *CLDN5* (95%), *SARP2* (91%), *reprimo* (91%), *LHX1* (77%), *FOXE1* (64%), *TJP2* (59%), *WNT7A* (59%), *CDH3*(18%),a nd *ST14* (9%). The number of aberrantly methylated genes varied among individual cell lines, with an average number of loci of 7.6 (range 4-11) per cell line (Figure 2). There was no significant correlation between the number of methylated loci and the genetic profile of cell lines with regard to the presence or absence of mutations in the K-*ras, p53,* and/or *SMADA4* genes.

EXAMPLE 6

Pattern of Changes in Expression of Genes Aberrantly Methylated in Pancreatic Cancer Cells

[0132] The relationship between methylation status of the genes identified as aberrantly methylated in pancreatic cancer with the change in expression levels after treatment with 5Aza-dC and/or TSA was then analyzed. The microarray data to compare the expression of the 11 genes among all the treatment groups in each of the four pancreatic cancer cell lines indicated that the most common pattern was weak or moderate induction after 5Aza-dC treatment, and strong (synergistic) induction after combined treatment (Figure 3). However, some of the genes were also induced by TSA treatment alone (e.g., *UCHL1* in Hs766T and Panc1). Furthermore, reexpression of genes completely methylated in an individual cell line was not observed after 5Aza-dC or combined treatment (e.g., *reprimo* in MiaPaCa2 and *SARP2* in Hs766T). It may be due to the primers used not covering the critical region or transcriptional regulation. It is also possible that other mechanisms besides DNA methylation and histone deacetylation could be involved in the silencing of these genes.

EXAMPLE 7

Methylation Analysis of Multiple Genes in Primary Pancreatic Cancers

[0133] To test whether the aberrant methylation of the genes identified in pancreatic cancer cell lines also occurred in primary pancreatic cancers, the methylation status of the 11 genes in 20 primary pancreatic cancers and 10 normal pancreatic ductal epithelia was analyzed. Aberrant methylation was also detected in 100% of primary pancreatic cancers for *UCHL1*, 100% of primary pancreatic cancers for *NPTX2*, 100% of primary pancreatic cancers for *SARP2*, 90% of primary pancreatic cancers for *CLDN5*, 85% of primary pancreatic cancers for *WNT7A*, 80% of primary pancreatic cancers for reprimo, 75% of primary pancreatic cancers for *LHX1*, 75% of primary pancreatic cancers for *FOXE1*, 70% of primary pancreatic cancers for *TJP2*, 20% of primary pancreatic cancers for *CDH3*, and 10% of primary pancreatic cancers for *ST14*. By contrast, 8 of 11 genes were completely unmethylated in all 10 of the samples from normal pancreatic ductal epithelia. Three genes (*UCHL1, WNT7A, and FOXE1*) showed a weak amplification of methylated alleles in 1-3 of the 10 normal pancreatic ductal epithelia. These results confirm the abnormal methylation patterns of these 11 genes in primary pancreatic carcinomas as well as in pancreatic cancer cell lines.

EXAMPLE 8

Methylation Analysis of Selected Genes in a Larger Panel of Primary Pancreatic Cancers and in Pancreatic Juice Samples

[0134] To test the diagnostic potential of genes identified as methylated in pancreatic cancer, three genes (*NPTX2, SARP2,* and *CLDN5*) were selected. These three genes were found to be frequently methylated in pancreatic cancer and not methylated in any of the normal ductal epithelia studied. To confirm the high prevalence of aberrant methylation at these loci, the methylation status of the genes in an expanded series of 43 surgically resected, primary pancreatic cancers, was analyzed. Aberrant methylation of *NPTX2, SARP2,* and *CLDN5* was detected in 42 (98%), 41 (95%), and 35 (81%) of these

43 primary pancreatic cancers (Figure 4), and hypermethylation of at least one of these loci was found in 100% of the primary tumors tested.

[0135] Pancreatic juice samples collected from patients with pancreatic cancer as described above, was then subject to analysis to determine whether aberrant hypermethylation of *NPTX2, SARP2,* and *CLDN5* could be detected. Using MSP, a total of 37 pancreatic juice samples including 24 samples from patients with pancreatic cancer and 13 samples from patients with benign pancreatic disorders were analyzed for the methylation patterns of the three genes. Aberrant methylation of *NPTX2* in 67%, of *SARP2* in 46%, and of *CLDN5* in 42% of the 24 juice samples from patients with pancreatic cancer. Overall, 75% (18 of 24) of juice samples from pancreatic cancer patients exhibited aberrant methylation of at least one gene, whereas none of the 13 juice samples from patients with benign pancreatic diseases harbored hypermethylation of any of these genes ($P<0.0001$). Importantly, aberrantly methylated DNA was detected in the five juice samples from patients with small pancreatic cancers (tumor < 2cm in diameter).

EXAMPLE 9

Summary

[0136] To identify potential targets for aberrant methylation in pancreatic cancer, gene expression profiles of four pancreatic cancer cell lines were analyzed after exposure to 5Aza-dC and/or TSA. A substantial number of genes were identified as having expression that was markedly induced by 5Aza-dC and TSA, either alone or in combination. Using MSP, the abnormal methylation patterns of 11 selected genes were confirmed in pancreatic cancer cells and in a series of resected primary pancreatic carcinomas. Most these genes have not been implicated as sites of aberrant methylation in any tumor type. These results demonstrate that gene expression profiling can be used to identify novel target genes that display aberrant methylation in pancreatic cancer. It was also demonstrated that these aberrantly methylated genes can be detected in a significant proportion of pancreatic juice samples from patients with pancreatic cancer

but not in juice samples from patients with benign pancreatic disorders. The results support that aberrant hypermethylation of multiple genes is a common event in pancreatic cancer and suggest that these genes can be sensitive and specific markers for the early detection of pancreatic cancer.

[0137] Recently, Suzuki et al. (*Nat. Genet.*, 31:141-149, 2002.) have described a cDNA microarray-based approach to screen for genes epigenetically silenced in colorectal cancer. They studied gene expression profiles in colorectal cancer cell line (RKO) treated with 5Aza-dC and/or TSA and successfully identified a number of genes harboring CpG island hypermethylation in colorectal cancer cell lines and in primary tumors; however, some of these genes were also methylated in normal colonic tissues. Although slight methylation was occasionally noted in only a small number of samples from normal pancreatic ductal epithelia, most of the genes identified as aberrantly methylated in pancreatic cancer were completely unmethylated in a panel of normal pancreatic ductal epithelia.

[0138] An advantage of using high-throughput oligonucleotide microarray data from multiple cell lines is the ability to identify a substantial number of candidates genes targeted for aberrant methylation in human cancers. Such data also provides the ability to conservatively estimate the number of genes directly affected by aberrant methylation in pancreatic cancers. Treatment of pancreatic cancer cell lines with 5Aza-dC induced an average of ~200 transcripts (range 116-251 transcripts) per cell line. Sixteen genes with CpG islands were selected from the list of genes induces by 5Aza-dC in pancreatic cancer cell lines but not in nonneoplastic HPDE cells, and it was confirmed that ~70% (11 of 16) of these genes were aberrantly methylated in pancreatic cancer. Therefore, an average of 140 genes (70% of 200 genes) may be aberrantly methylated in a pancreatic cancer cell line, of which 60 would be expected to be CpG islands [one previous study has estimated that 60% of genes induced by 5Aza-dC do not have CpG islands within their 5' regions]. Sixty aberrantly methylated CpG islands in a pancreatic cancer is considered to be a minimum estimate for several reasons: analysis did not include a large fraction of ESTs on the Affymetrix U133B chip; expression of many genes that harbor

aberrantly methylated CpG islands may be unaffected by 5Aza-dC treatment; and because of the use of a stringent 5-fold induction of expression as a cutoff for identifying genes induced by 5Aza-dC.

[0139] Previously, Costello et al. (*Nat. Genet.*., 24:132-138, 2000.) studied a pair of cancers using RLGS and estimated that ~600 CpG islands were aberrantly methylated in a given cancer. Their estimate is higher than ours for a number of reasons. RLGS also identifies methylated CpG islands that are unrelated to genes (~22% of CpG islands in their study). Treatment with 5Aza-dC induced the expression of only one-third of the CpG islands they identified as hypermethylated in tumors. In addition, RLGS may also identify methylated CpG islands in tumors when corresponding normal tissue has a low level methylation. This study and results highlight the fact that in human cancers, a substantial number of genes are silenced by aberrant methylation. Similarly, the large number of genes induced by TSA is consistent with previous reports that have found between 2% and 10% of genes are induced in cancer cells by TSA treatment.

[0140] Variability was observed in the gene expression response of individual cell lines to 5Aza-dC and to TSA. Some cell lines harboring methylation of CpC islands at a specific locus had induction of gene expression after 5Aza-dC treatment, whereas others did not. The same observation was true for TSA treatment. Surprisingly, none of the genes analyzed in this study showed induction (> 5-fold) after 5Aza-dC treatment in all of the four pancreatic cancer cell lines, even when a gene was methylated in each of these cell lines. This may partly reflect the use of a 5-fold cutoff as an indicator of a significant induction of expression. It is also likely that differences in CpG island methylation density and different levels of transcriptional cofactors between different cell lines contribute to differences in gene expression responses to 5Aza-dC and to TSA. Because a panel of genes induced by 5Aza-dC treatment in even one of the four pancreatic cancer cell lines tested usually led to identifying aberrant CpG methylation of these genes in other pancreatic cancer cell lines, it proved helpful to provide a list of all genes induced 5-fold or greater by 5Aza-dC treatment.

[0141] A number of genes without 5' CpG islands were identified that were up-regulated after 5Aza-dC treatment. These findings imply that even genes with poor CpG promoters can be regulated by DNA methylation. In keeping with this notion, it was observed that relatively CpG-poor genes such as 14-3-3σ are aberrantly methylated in cancer. Genes induced by 5Aza-dC that are known to be overexpressed in pancreatic and other cancers (for example, *kallikrein 10*) were also identified. Interestingly, *kallikrein 10* has previously been shown to be methylated in certain cancers. This observation suggests that alterations in methylation patterns may be responsible for the overexpression, as well as the underexpression, of many affected genes in cancer.

[0142] One of the novel findings in this study is that TSA alone could induce the expression of 4 of the 11 genes whose CpG islands were identified as aberrantly methylated in pancreatic cancer. In addition, several genes previously characterized as having methylated CpG islands (such as *p57KIP2* and *CACNA1G*) were also reexpressed after treatment with TSA alone. Previous studies have found that TSA alone is not sufficient to induce the expression of genes with densely methylated CpG islands, although it can facilitate induction of gene expression when combined with 5Aza-dC. Recently El-Osta et al. (*Mol. Cell. Biol.*, 22:1844-1857, 2002) have reported that methyl-CpG-binding protein 2 is involved in methylation-dependent silencing of the *MDR1* gene and that treatment with 5-azacytidine but not TSA can release methyl-CpG-binding protein 2 from the heavily methylated promoter, thereby leading to a partial relief of the transcriptional repression. Although the mechanisms underlying the correlation between DNA methylation and histone deacetylation in the control of gene expression are still under investigation, our results provide evidence that treatment with TSA alone can, at least in some cases, relieve the silencing of methylated genes in cancer cells.

[0143] Changes in methylation patterns play a crucial role in cancer development and progression. A number of genes identified herein as aberrantly methylated in pancreatic cancer have known important properties involved in cell cycle regulation (*reprimo*), apoptosis (*SARP2*), cell adhesion (*CDH#*), and tight junction barrier (*CLDN5* and *TJP2*). Aberrant methylation and associated silencing of these genes may be

functionally important for pancreatic carcinogenesis. For example, *reprimo*, which displayed frequent hypermethylation in pancreatic cancer, is a downstream mediator of p53-induced G_2 cell cycle arrest. When overexpressed, *reprimo* induces cell cycle arrest at the G_2 phase, suggesting it has tumor suppressor function. Because functional abrogation of the *p53* tumor suppressor gene and its downstream mediators, such as *14-3-3σ*, is central to the development of human cancers, it is likely that aberrant methylation of *reprimo* could lead to defects in cell cycle control and contribute to pancreatic neoplastic progression.

[0144] It is also shown that *SARP2* is a frequent target for aberrant methylation in pancreatic cancer. *SARP2* is a member of *SARP* gene families that counteract the Wnt oncogenetic signaling pathway, and this gene is considered to be involved in the regulation of apoptosis. Breast cancer cells transfected with *SARP2* show an increased sensitivity to different proapoptotic stimuli. Therefore, inactivation of *SARP2* by aberrant methylation may provide a growth advantage to cancer cells through increasing the cellular resistance to apoptosis. Interestingly, *SARP2* ahs recently been identified as frequently hypermethylated in colorectal and gastric cancer, thus suggesting general involvement of this gene in tumorigenesis of digestive organs.

[0145] Although a growing number of genes have been identified as aberrantly methylated in various cancers, to date few genes have been reported that are aberrantly methylated in a large majority of cancers. The approach taken herein identified five genes (*UCHL1, NPTX2, SARP2, CLDN5*, and *reprimo*), each of which was aberrantly methylated in > 80% of a panel of pancreatic cancer cell lines. Furthermore, all of the genes found to be methylated in pancreatic cancer cell lines were also methylated in primary pancreatic carcinomas. This supports the observation made herein that aberrantly methylated genes identified in cancer cell lines are often present in the primary cancers from which they were derived. Genes that are aberrantly methylated at a high frequency in a given cancer are particularly suitable for early cancer detection strategies.

[0146] Several studies have addressed the diagnostic utility of epigenetic markers in detection of cancer. Methylation abnormalities have been detected in blood or sputum of patients with lung cancer, in serum of patients with head and neck cancer, in ductal lavage fluid of patients with breast cancer, and in urine from patients with prostate and bladder cancer. In particular, the inclusion of multiple genes in these analyses appears to provide a highly sensitive and specific marker for cancer diagnosis. Using three markers, it was possible to detect aberrantly methylated DNA in 75% of pancreatic juice samples from patients with pancreatic cancer.

[0147] Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

TABLE 1

Fold-change after 5Aza-dC treatment					Induced by TSA alone?	CpG island?	Genbank#	Gene name (symbol)
AsPC1	Hs766T	MiaPaca	Panc1	HPDE				
6.15	-0.44	2.27	0.91	-0.4	No	No	NM_001147	angiopoietin 2 (ANGPT2)
6.12	-1.75	21.32	-1.07	1.23	Yes	No	NM_004052	BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3)
1.97	1.36	8.59	-1.04	-1.06	No	No	NM_004335	bone marrow stromal cell antigen 2 (BST2)
-1.07	5.21	-1.3	-1.01	2.23	No	Yes	NM_000465	BRCA1 associated RING domain 1 (BARD1)
1.44	0.89	5.79	2.31	-1.08	No	Yes	NM_001793	cadherin 3, type 1, P-cadherin (placental) (CDH3)
1.66	6.65	-1.09	1.03	1.06	No	Yes	NM_019845	candidate mediator of the p53-dependent G2 arrest (reprimo)
22.34	1.21	1.67	1.88	2.45	No	Yes	NM_003277	claudin 5 (CLDN5)
1.12	5.75	4.23	-1.91	-1.03	Yes	Yes	M76453	colony stimulating factor 1 (CSF1) gene
1.48	5.28	1.09	1.52	1.5	No	Yes	NM_004702	cyclin E2 (CCNE2)
2.21	1.04	7.04	1.93	1.33	No	Yes	NM_000389	cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A)
0.6	5.42	2.28	3.77	1.07	Yes	Yes	NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C)
1.47	-1.46	2.12	5.19	1.01	No	Yes	U72763	death receptor 3 (DR3)
3.51	5.63	3.8	1.7	-1.1	No	Yes	NM_004473	forkhead box E1 (thyroid transcription factor 2) (FOXE1)
-0.54	0.48	6.7	0.46	-0.55	Yes	Yes	NM_001468	G antigen 1 (GAGE1)
7.55	-2.57	15.22	9.78	1.33	Yes	No	NM_001473	G antigen 3 (GAGE3)
15.66	-1.1	47.6	35.44	3.33	Yes	Yes	NM_001474	G antigen 4 (GAGE4)
15.59	1.09	24.03	35.5	2.41	Yes	Yes	NM_001476	G antigen 6 (GAGE6)
14.08	-1.12	54.35	36.1	3.36	Yes	Yes	NM_021123	G antigen 7 (GAGE7)
1.44	5.53	3.97	2.17	-0.94	Yes	No	M11734	granulocytemacrophage colony-stimulating factor
25.8	-1.61	2.56	1.26	-1.23	No	Yes	S79910	HOXA1
0.55	-0.41	0.81	9.41	-0.34	No	Yes	NM_000612	insulin-like growth factor 2 (somatomedin A) (IGF2)
-1.12	1.02	21.28	-0.84	0.89	No	No	NM_002201	interferon stimulated gene (20kD) (ISG20)
4.48	-4.46	11.03	1.2	1.33	Yes	No	NM_022873	interferon, alpha-inducible protein
7.51	-2.59	-1.77	1.37	-1.81	No	No	NM_000877	interleukin 1 receptor, type I (IL1R1)
1.27	5.27	1.4	-4.18	2.14	No	Yes	NM_020655	junctophilin 3 (JPH3)
1.03	1.16	136.95	-1.05	3.75	No	No	BC002710	kallikrein 10 (KLK10)
8.99	-1.86	-1.01	9.19	-1.12	No	Yes	NM_012317	leucine zipper, down-regulated in cancer 1 (LDOC1)
5.22	1.92	1.89	1.79	1.01	No	Yes	NM_005568	LIM homeobox protein 1 (LHX1)
1.52	4.45	2.27	7.32	1.7	Yes	Yes	NM_030801	MAGE1 protein (MAGE1)
-1.44	6.83	-1.01	3.61	-1.25	Yes	No	NM_002379	matrilin 1, cartilage matrix protein (MATN1)
2.94	-1.24	6.73	4.59	-1.08	Yes	Yes	NM_021076	neurofilament, heavy polypeptide (200kD) (NEFH)
13.97	-2.6	6.4	18.94	2.42	Yes	Yes	U26662	neuronal pentraxin II (NPTX2)
-1.22	1.04	5.45	1.04	-1.58	No	Yes	NM_006034	p53-induced protein (PIG11)
8.94	1.36	-1.12	4.45	2.57	No	Yes	NM_000280	paired box gene 6 (aniridia, keratitis) (PAX6)
-1.15	-1.73	6.4	2.13	4.9	No	No	NM_000930	plasminogen activator, tissue (PLAT)
1.37	1.12	7.95	1.08	1.2	No	Yes	NM_016445	pleckstrin 2 (mouse) homolog (PLEK2)
1.33	6.38	3.48	4.84	-1.04	No	No	NM_024411	prodynorphin (PDYN)
-1.89	1.35	38.33	-1.47	1.11	No	Yes	AF017987	secreted apoptosis related protein 2 (SARP2)
-1.48	-1.64	34.77	1.21	-1.46	Yes	Yes	BC000329	stratifin (14-3-3 sigma)
14.06	1.01	4.96	6.84	4.8	Yes	Yes	NM_012447	stromal antigen 3 (STAG3)
-1.21	-4.72	1.48	5.1	-1.05	Yes	Yes	NM_021978	suppression of tumorigenicity 14 (colon carcinoma, matriptase, epithin) (ST14)
7.89	-0.79	-1.07	1.01	3.54	No	Yes	NM_003069	SWISNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (SMARCA1)
-1.23	1.6	9.61	1.57	1.09	Yes	Yes	BC001451	testin
-1.24	-1.56	8.61	1.49	1.41	No	Yes	NM_004817	tight junction protein 2 (zona occludens 2) (TJP2)
11.89	3.67	1	1.1	1.32	No	Yes	NM_000362	tissue inhibitor of metalloproteinase 3 (TIMP3)
-1.17	-4.9	31.63	1.18	-1.2	No	No	NM_000594	tumor necrosis factor (TNF superfamily, member 2) (TNF)
6.62	1.45	5.27	2.37	1.95	Yes	Yes	NM_004181	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) (UCHL1)
1.83	0.5	1.61	5.26	0.39	Yes	Yes	AF126966	voltage-dependent calcium channel alpha 1G subunit a isoform (CACNA1G)
2.7	9.03	6.24	3.57	-1.23	Yes	Yes	D83175	wingless-type MMTV integration site family, member 7A (WNT7A)
28.59	-0.5	12.99	24.78	-0.43	Yes	Yes	NM_020411	XAGE-1 protein (XAGE-1)

TABLE 2

Fold-change after TSA treatment				Induced by 5AzadC alone?	CpG island?	Genbank#	Gene name (symbol)
AsPC1	Hs766T	MiaPaCa	Panc1				
8.73	1.73	2.14	2.74	No	Yes	NM_001674	activating transcription factor 3 (ATF3)
7.6	1.86	5.02	3.22	No	Yes	NM_001124	adrenomedullin (ADM)
18.35	2.02	3.97	4.2	No	Yes	NM_001657	amphiregulin (schwannoma-derived growth factor) (AREG)
-1.77	6.72	1.75	-1.13	No	No	NM_001146	angiopoietin 1 (ANGPT1)
1.65	2.35	10.05	5.02	No	Yes	NM_001197	BCL2-interacting killer (apoptosis-inducing) (BIK)
1.11	-1.11	-1.29	6.85	Yes	Yes	AB035305	cadherin-8 (CDH8)
1.73	1.06	5.18	5.41	No	Yes	NM_004055	calpain 5 (CAPN5)
-1.47	5.14	1.15	1.01	No	Yes	AF040708	candidate tumor suppressor gene 21 protein
13.22	11.83	13.25	1.5	No	Yes	AF044076	candidate tumor suppressor p33ING1 (ING1)
-1.39	1.82	3.22	11.66	No	Yes	NM_006569	cell growth regulatory with EF-hand domain (CGR11)
1.87	-1.11	-1.03	9.31	No	Yes	NM_005197	checkpoint suppressor 1 (CHES1)
-1.1	8	1.27	-1.33	No	Yes	NM_018223	checkpoint with forkhead and ring finger domains (CHFR)
-4.26	-1.81	1.13	8.69	No	No	NM_014343	claudin 15 (CLDN15)
17.38	43	4.28	7.69	No	Yes	M92934	connective tissue growth factor
10.62	6.57	13.22	6.26	Yes	Yes	NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C)
4.56	-1.26	5.91	-2.06	Yes	Yes	NM_004426	early development regulator 1 (homolog of polyhomeotic 1) (EDR1)
37.6	6.49	8.67	6.44	No	Yes	NM_001965	early growth response 4 (EGR4)
1.91	7.43	1.7	-1.37	No	Yes	NM_001432	epiregulin (EREG)
0.66	14.57	0.53	0.63	Yes	Yes	NM_001468	G antigen 1 (GAGE1)
-2.5	432.64	1.31	1.4	No	No	NM_001472	G antigen 2 (GAGE2)
-5.88	184.93	-2.68	1.07	Yes	Yes	NM_001473	G antigen 3 (GAGE3)
-1.67	234.36	1.32	2.29	Yes	Yes	NM_001474	G antigen 4 (GAGE4)
-1.16	237.45	-2.17	1.62	Yes	Yes	NM_001476	G antigen 6 (GAGE6)
-4.88	331.97	-1.76	3.56	Yes	Yes	NM_001477	G antigen 7B (GAGE7B)
2.65	2.01	5.06	-1.09	No	Yes	AF087853	growth arrest and DNA damage inducible protein beta (GADD45B)
6.64	5.8	4.16	5.04	No	Yes	BC002649	H1 histone family, member 2
1.73	2.56	5.25	1.21	No	Yes	NM_002133	heme oxygenase (decycling) 1 (HMOX1)
28.5	1.55	77.77	4.55	Yes	Yes	NM_000558	hemoglobin, alpha 1 (HBA1)
2.18	-1.12	14.09	3.03	No	Yes	NM_006665	heparanase (HPSE)
10.26	5	2.27	21.22	No	Yes	NM_002166	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2)
44.67	-1.42	1.36	3.01	No	No	NM_000584	interleukin 8 (IL8)
7.63	1.54	2.91	2.13	Yes	No	NM_019598	kallikrein 12 (KLK12)
1.05	0.49	5.17	15.48	Yes	Yes	NM_030801	MAGE1 protein (MAGE1)
9.05	1.63	1.01	-2.63	No	No	NM_002421	matrix metalloproteinase 1 (interstitial collagenase) (MMP1)
2.66	7.94	1.28	-1.82	No	Yes	NM_015845	methyl-CpG binding domain protein 1 (MBD1)
0.76	4.11	13.46	30.7	Yes	Yes	NM_002507	nerve growth factor receptor (TNFR superfamily, member 16) (NGFR)
7.93	2.06	-1.04	3.9	No	Yes	NM_000435	Notch (Drosophila) homolog 3 (NOTCH3)
20.29	9.7	1.28	-3.83	Yes	Yes	NM_002616	period (Drosophila) homolog 1 (PER1)
1.08	5.74	2.74	3.69	No	Yes	AF016535	P-glycoprotein (mdr1)
4.45	3.17	16.4	4.05	Yes	Yes	AF003934	prostate differentiation factor
8	1.95	44.44	11.7	No	Yes	NM_005025	protease inhibitor 12 (neuroserpin) (SERPINI1)
413.57	16.31	26.21	31.64	No	Yes	NM_005794	short-chain alcohol dehydrogenase family member (HEP27)
-0.78	15.92	-1.34	6.45	No	No	NM_013453	sperm protein associated with the nucleus, X chromosome, family member A1 (SPANXA1)
15.9	-1.31	2.73	5.63	Yes	No	NM_003155	stanniocalcin 1 (STC1)
1.74	38.74	1.5	2.73	Yes	No	BC001003	synovial sarcoma, X breakpoint 1
0.4	120.09	-0.68	34.65	No	Yes	L27624	tissue factor pathway inhibitor-2
73.47	3.47	4.09	18.25	Yes	Yes	NM_025217	UL16-binding protein 2 (ULBP2)
5.48	-1.45	6.39	2.05	No	Yes	U94592	uncoupling protein homolog (UCPH)
9.95	2.72	4.61	10.14	Yes	Yes	AF126966	voltage-dependent calcium channel alpha 1G subunit a isoform (CACNA1G)
5.72	0.81	3.36	5.13	Yes	Yes	NM_030761	wingless-type MMTV integration site family, member 4 (WNT4)

TABLE 3

Fold-change after combined treatment				Induced by 5AzadC alone?	Induced by TSA alone?	Genbank#	Gene name (symbol)
AsPC1	Hs766T	MiaPaCa	Panc1				
11.06	8.03	1.08	1.19	No	Yes	AF193421	activity-regulated cytoskeleton-associated protein (APC)
2.96	8.04	4.8	2.13	No	Yes	AB017332	AuroraIpl1-related kinase 3 (aik3)
-1.5	13.56	2.23	-1.31	No	Yes	M27968	basic fibroblast growth factor (FGF)
9.73	8.23	10.27	-1.08	Yes	Yes	U15174	BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3)
2.07	8.35	14.61	3.72	No	Yes	NM_001197	BCL2-interacting killer (apoptosis-inducing) (BIK)
2.97	1.88	8.02	-1.28	Yes	No	NM_004335	bone marrow stromal cell antigen 2 (BST2)
5.59	-1.22	1.28	1.13	No	No	NM_004347	caspase 5, apoptosis-related cysteine protease (CASP5)
-2.26	5.18	5.02	1.47	No	No	BC005406	Cdc42 effector protein 2
2.45	5.04	1.44	1.58	No	No	NM_001789	cell division cycle 25A (CDC25A)
1.89	8.56	4.04	1.18	No	No	NM_001307	claudin 7 (CLDN7)
-2.5	6	4.31	-1.59	No	No	NM_000759	colony stimulating factor 3 (granulocyte) (CSF3)
17.6	1.59	7.11	30.71	No	No	NM_003914	cyclin A1 (CCNA1)
-3.01	6.9	1.11	-1.55	No	No	NM_000077	cyclin-dependent kinase inhibitor 2A (melanoma, p16) (CDKN2A)
-2.28	20.41	12.58	1.16	No	Yes	NM_001311	cysteine-rich protein 1 (intestinal) (CRIP1)
20.29	0.61	2.14	12.03	No	Yes	NM_004750	cytokine receptor-like factor 1 (CRLF1)
5.85	0.41	2.83	-1.06	No	No	AF199015	cytovillin 2 (VIL2, ezrin)
6.41	3.45	-0.34	0.62	No	No	AY004154	DEAD box RNA helicase (VASA)
5.06	1.62	-1.36	-3.25	No	No	NM_014326	death-associated protein kinase 2 (DAPK2)
52.76	91.78	41.45	59.31	Yes	Yes	NM_001351	deleted in azoospermia-like (DAZL)
38.06	405.04	185.65	141.23	Yes	Yes	NM_001472	G antigen 2 (GAGE2)
26.49	275.96	149.24	129.27	No	Yes	NM_001477	G antigen 7B (GAGE7B)
0.49	5.02	-1.98	0.4	No	No	NM_002048	growth arrest-specific 1 (GAS1)
1.07	116.32	-1.01	-1.03	No	Yes	NM_005345	heat shock 70kD protein 1A (HSPA1A)
5.49	-1.1	2.39	1.55	No	Yes	NM_030926	integral membrane protein 3 (ITM3)
1.4	5.57	10.06	1.29	No	Yes	AF154005	junction adhesion molecule
7.42	4.37	3.91	0.42	No	No	NM_000422	keratin 17 (KRT17)
6.69	6.07	2.39	0.55	Yes	No	NM_004789	LIM homeobox protein 2 (LHX2)
-0.49	-0.49	10.62	0.41	Yes	No	NM_000236	lipase, hepatic (LIPC)
6.52	1.72	2.32	-1.18	No	No	NM_002424	matrix metalloproteinase 8 (neutrophil collagenase) (MMP8)
8.46	7.88	5.95	4.31	No	Yes	NM_005462	melanoma antigen, family C, 1 (MAGEC1)
1.16	5.37	1.03	1.02	No	Yes	NM_000249	mutL (E. coli) homolog 1 (colon cancer, nonpolyposis type 2) (MLH1)
14.18	1.67	2.89	1.09	Yes	Yes	NM_004221	natural killer cell transcript 4 (NK4)
5.1	10.46	16.4	14.69	No	Yes	AF298547	nucleotide-binding site protein 1
6.8	4.51	6	1.05	Yes	No	NM_006034	p53-induced protein (PIG11)
12.42	7.96	-1.27	1.22	Yes	No	NM_000280	paired box gene 6 (aniridia, keratitis) (PAX6)
10.36	18.77	1.09	-1.11	No	Yes	NM_002727	proteoglycan 1, secretory granule (PRG1)
9.43	-1.39	11.73	4.76	Yes	No	NM_005978	S100 calcium-binding protein A2 (S100A2)
3.61	-0.61	7.64	18.09	No	No	NM_005980	S100 calcium-binding protein P (S100P)
9.24	1.83	9.38	2.08	Yes	No	NM_002968	sal (Drosophila)-like 1 (SALL1)
3.64	7.02	1.39	-1.36	No	Yes	NM_003919	sarcoglycan, epsilon (SGCE)
1.57	-1.69	19.85	-1.39	Yes	No	AF017987	secreted apoptosis related protein 2 (SARP2)
5.33	1.87	1.12	1.07	No	Yes	BC000627	Signal transducer and activator of transcription 3 (STAT3)
29.63	9.56	1.23	6.25	No	Yes	NM_022661	SPANX family, member C (SPANXC)
17.75	11.08	27.2	8.21	No	Yes	AF257500	synovial sarcoma, X breakpoint 2
5.4	0.41	1.24	-1.23	No	No	NM_003219	telomerase reverse transcriptase (TERT)
468.96	60.57	104.53	146.17	No	Yes	X91817	transketolase-like protein (2418 bp)
17.87	11.04	6.91	6.89	Yes	Yes	NM_004181	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) (UCHL1)
2.2	1.34	8.91	-1.16	Yes	No	NM_015855	Wilms tumor associated protein (WIT-1)
6.52	1.23	-1.23	4.4	No	No	NM_003881	WNT1 inducible signaling pathway protein 2 (WISP2)
35.75	89.1	20.74	38.95	Yes	Yes	NM_020411	XAGE-1 protein (XAGE-1)

TABLE 4

Gene		Primer sequences	
cadherin 3 (CDH3)	Unmethylated	F	TGT GTG TGG GAG GAT GTA TG
		R	ACA CAT CTA AAA TCA ACT AAA AAC
	Methylated	F	GCG TGT GGG AGG ACG TAC
		R	ACA TCT AAA ATC GAC TAA AAA CG
reprimo	Unmethylated	F	TTG TGA GTG AGT GTT TAG TTT G
		R	TAA TTA CCT AAA ACC AAA TTC ATC
	Methylated	F	GCG AGT GAG CGT TTA GTT C
		R	TAC CTA AAA CCG AAT TCA TCG
claudin 5 (CLDN5)	Unmethylated	F	TGA TAG ATT TGT GGG GTA AAT G
		R	CCC AAA ACC ATA CTA CAC AAC
	Methylated	F	GAT AGA TTC GCG GGG TAA AC
		R	TAC GCG ACG CCC TAA ACG
death receptor 3 (DR3)	Unmethylated	F	GTT AGG TGG GTT TTT TTT GAT G
		R	TCC ATA ACC CTC CAA CAA AC
	Methylated	F	TTA GGC GGG TTT TTT TCG AC
		R	CAT AAC CCT CCG ACG AAC G
forkhead box E1 (FOXE1)	Unmethylated	F	TTT GTA GGG TTG GAG ATT TAT G
		R	AAA ACA AAA CAA AAA CAA CAA AAT C
	Methylated	F	TCG TAG GGT TGG AGA TTT AC
		R	GAA ACG AAA ACA ACG AAA TCG
leucine zipper down-regulated in cancer 1 (LDOC1)	Unmethylated	F	TTA TTA AGT GTT TTT GTG GAT ATG
		R	CCT AAA AAA ACA AAA CTC AAC TC
	Methylated	F	TAT TAA GCG TTT TCG TGG ATA C
		R	CGA CCT AAA AAA ACA AAA CTC G
LIM homeobox protein 1 (LHX1)	Unmethylated	F	GTG TTT TTT TTG TAA TTT GAG TTT G
		R	AAC CCA CAA AAA AAT AAA AAT CAA C
	Methylated	F	GTT TTT TTC GTA ATT CGA GTT C
		R	CGC GAA AAA ATA AAA ATC AAC G
neurofilament heavy polypeptide (NEFH)	Unmethylated	F	GGT GGT GGT AGT TTT TAT TAT G
		R	ATC CAT ATC CAC AAA TAA AAA CC
	Methylated	F	GCG GCG GTA GTT TTT ATT AC
		R	AAA CCG CTA AAA AAA CCA ACG
neuronal pentraxin II (NPTX2)	Unmethylated	F	AAG AAA GGG TGT GTG GAT TTG
		R	CCA CAC TAT CAT CTC AAA AAT C
	Methylated	F	GAA AGG GCG CGC GGA TTC
		R	CGC TAT CGT CTC GAA AAT CG
p53-induced protein (PIG11)	Unmethylated	F	GTT TGG GTT TGG GAG ATA TTG
		R	CCC CTT TCT TAA AAC AAT CAA C
	Methylated	F	GTT TGG GTT CGG GAG ATA TC
		R	CCT TTC TTA AAA CGA TCA ACG
secreted apoptosis related protein 2 (SARP2)	Unmethylated	F	GGG TGT ATT TAG TTT GTA GTG
		R	CAA TCC CCC CAA CCA ATA AC
	Methylated	F	GTC GGG GCG TAT TTA GTT C
		R	TCC CCC CGA CCA ATA ACG
suppression of tumorigenicity 14 (ST14)	Unmethylated	F	GTG TTA GGG TGA GGG TAT TG
		R	ACT CAC AAA TCT CAC AAA CAT C
	Methylated	F	CGT TAG GGC GAG GGT ATC
		R	CTC GCG AAT CTC ACA AAC G

TABLE 4 (continued)

SWI/SNF -related gene (*SMARCA1*)	Unmethylated	F	TGT GGA TGT GAT TGT TAT TAT TG
		R	TCT CCA TAA CCA CAA TAA CTT C
	Methylated	F	CGG ATG CGA TCG TTA TTA TC
		R	CGT AAC CGC GAT AAC TTC G
tight junction protein 2 (*TJP2*)	Unmethylated	F	TTG TGG GTT AGA GTA TTG TTT G
		R	ATC TCC TCA CAC CAA CAT TTC
	Methylated	F	TGC GGG TTA GAG TAT TGT TC
		R	CCT CGC ACC GAC ATT TCG
ubiquitin carboxyl-terminal esterase L1 (*UCHL1*)	Unmethylated	F	TTT GTA TTT ATT TGG TTG TGA TTG
		R	CCC AAA CTA CAA CTA TAA AAC ACC
	Methylated	F	TTA TTT GGT CGC GAT CGT TC
		R	CCC AAA CTA CAA CTA TAA AAC G
wingless-type MMTV integration site family, member 7A (*WNT7A*)	Unmethylated	F	TAG TTT GGT GTT GTT TTA TGT TG
		R	CCC CAA AAC CAT CTA TCA ATA C
	Methylated	F	GTA GTT CGG CGT CGT TTT AC
		R	CGA AAC CGT CTA TCG ATA CG

What is claimed is:

1. A method for detecting pancreatic carcinoma in a subject comprising:

a) contacting a nucleic acid-containing specimen from the subject with an agent that provides a determination of the methylation state of at least one gene or associated regulatory region of the gene; and

b) identifying aberrant methylation of regions of the gene or regulatory region, wherein aberrant methylation is identified as being different when compared to the same regions of the gene or associated regulatory region in a subject not having the pancreatic carcinoma, thereby detecting pancreatic carcinoma in the subject.

2. The method of claim 1, wherein the regions of the gene are contained within CpG rich regions.

3. The method of claim 1, wherein the gene is selected from at least one gene listed in Tables 1-3, or any combination thereof.

4. The method of claim 1, wherein aberrant methylation comprises hypermethylation when compared to the same regions of the gene or associated regulatory regions in a subject not having pancreatic carcinoma.

5. The method of claim 4, wherein the regions comprise regulatory regions of the gene.

6. The method of claim 1, wherein the agent is a pair of primers that hybridize with a target sequence in the gene or associated regulatory region of the gene.

7. The method of claim 1, wherein the nucleic acid-containing specimen is selected from pancreatic ductal epithelium, pancreatic juice, or pancreatic tissue.

8. The method of claim 7, wherein the nucleic acid-containing specimen is pancreatic fluid.

9. The method of claim 1, wherein the pancreatic carcinoma is selected from pancreatic ductal adenocarcinoma, chronic pancreatitis, islet cell tumor, or serus cystadenoma.

10. A method of identifying at least one gene silenced by DNA methylation associated with pancreatic carcinoma, comprising:

a) contacting an array of nucleotide sequences representative of a genome with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of silenced genes but not RNA expressed in normal cells corresponding to the cancer cells, under conditions suitable for selective hybridization of nucleic acid subtraction products to complementary nucleotide sequences of the array; and

b) detecting selective hybridization of nucleic acid to a subpopulation of nucleotide sequences of the array,

wherein nucleic acid molecules corresponding to RNA expressed in the normal cells corresponding the cancer cells do not hybridize to the subpopulation of nucleotide sequences under the conditions suitable for selective hybridization,

whereby the nucleic acid molecules that selectively hybridize to the subpopulation of nucleotide sequences of the array represent epigenetically silenced genes of the cancer cells, thereby identifying at least one epigenetically silenced gene associated with pancreatic cancer.

11. The method of claim 10, wherein the nucleic acid molecules corresponding to RNA comprise cDNA.

12. The method of claim 10, wherein the at least one agent comprises 5 aza 2' deoxycytidine (5Aza-dC).

13. The method of claim 10, wherein the at least one agent comprises trichostatin (TSA).

14. The method of claim 10, wherein the at least one agent comprises a combination of 5 aza 2' deoxycytidine (5Aza-dC) and trichostatin (TSA).

15. The method of claim 10, wherein the at least one methylation silenced gene is associated with pancreatic cancer.

16. The method of claim 10, wherein the at least one methylation silenced gene is selected from a gene listed in Tables 1-3, or any combination thereof.

17. The method of claim 10, wherein the at least one methylation silenced gene is selected from CDH3, reprimo, CLDN5, DR3, FOXE1, LDOC1, LHX1, NEFH, NPTX2, PIG11, SARP2, ST14, SMARCA1, TJP2, UCHL1, WNT7A, or a combination thereof.

18. The method of claim 10, wherein the at least one cancer is a carcinoma or a sarcoma.

19. An amplification primer pair, comprising a forward primer and a reverse primer as set forth in SEQ ID NOS: 1 to 64, wherein the amplification primer pair can amplify a portion of a gene of Tables 1-3.

20. The amplification primer pair of claim 19, which can specifically amplify a methylated 5' regulatory region of the nucleic acid molecule.

21. The amplification primer pair of claim 20, comprising SEQ ID NOS: 3 and 4, SEQ ID NOS: 7 and 8, SEQ ID NOS: 11 and 12, SEQ ID NOS: 15 and 16, SEQ ID NOS: 19 and 20, SEQ ID NOS: 23 and 24, SEQ ID NOS: 27 and 28, SEQ ID NOS: 31 and 32, SEQ ID NOS: 35 and 36, SEQ ID NOS: 39 and 40, SEQ ID NOS: 43 and 44,

SEQ ID NOS: 47 and 48, SEQ ID NOS: 51 and 52, SEQ ID NOS: 55 and 56, SEQ ID NOS: 59 and 60 or SEQ ID NOS: 63 and 64.

22. An amplification primer pair of claim 19, which can specifically amplify a unmethylated 5' regulatory region of the nucleic acid molecule.

23. The amplification primer pair of claim 22 comprising SEQ ID NOS: 1 and 2, SEQ ID NOS: 5 and 6, SEQ ID NOS: 9 and 10, SEQ ID NOS: 13 and 14, SEQ ID NOS: 17 and 18, SEQ ID NOS: 21 and 22, SEQ ID NOS: 25 and 26, SEQ ID NOS: 29 and 30, SEQ ID NOS: 33 and 34, SEQ ID NOS: 37 and 38, SEQ ID NOS: 41 and 42, SEQ ID NOS: 45 and 46, SEQ ID NOS: 49 and 50, SEQ ID NOS: 53 and 54, SEQ ID NOS: 57 and 58, or SEQ ID NOS: 61 and 62.

24. A kit containing at least one amplification primer pair comprising a methylation specific amplification primer pair, an unmethylation specific amplification primer pair, or a combination comprising at least one methylation specific amplification primer pair and at least one unmethylation specific amplification primer pair.

25. The kit of claim 24, further comprising a reagent that modifies methylated cytosine residues.

26. The kit of claim 24, further comprising a methylation sensitive restriction endonuclease.

27. The kit of claim 24, further comprising reagents for performing an amplification reaction.

28. A kit useful for the detection of pancreatic carcinoma in a subject comprising:

a) carrier means compartmentalized to receive a sample therein;

b) one or more containers comprising a first container containing a reagent which modifies unmethylated cytosine and a second container containing primers for amplification of a CpG-containing nucleic acid, wherein the primer is selected from SEQ ID NO:1-64.

29. The kit of claim 28, further comprising a third container containing a methylation sensitive restriction endonuclease.

30. The kit of claim 28, wherein the modifying reagent is bisulfite.

31. The kit of claim 28, wherein the primer hybridizes with a target gene as set forth in Tables 1-3.

32. A method for monitoring a therapeutic regimen for treating a subject having pancreatic cancer comprising:

a) obtaining a nucleic acid-containing specimen from the subject prior to therapy;

b) contacting the nucleic acid-containing specimen with an agent that provides a determination of the methylation state of at least one gene or associated regulatory region of the gene;

c) identifying aberrant methylation of regions of the gene or regulatory region, wherein aberrant methylation is identified as being different when compared to the same regions of the gene or associated regulatory region in a subject not having the pancreatic cancer; and

d) determining a change in the methylation state of the gene during therapy, wherein the change is determined by comparing the methylation state of the gene with the methylation state of the same gene from the nucleic acid-containing sample from the same subject prior to therapy, thereby monitoring a therapeutic regimen for treating a pancreatic cancer subject.

33. The method of claim 32, wherein the regions of the gene are contained within CpG rich regions.

34. The method of claim 32, wherein the gene is selected from at least one gene listed in Tables 1-3, or any combination thereof.

35. The method of claim 32, wherein aberrant methylation comprises hypermethylation when compared to the same regions of the gene or associated regulatory regions in a subject not having pancreatic carcinoma.

36. The method of claim 35, wherein the regions comprise regulatory regions of the gene.

37. The method of claim 32, wherein the agent is a pair of primers that hybridize with a target sequence in the gene or associated regulatory region of the gene.

38. The method of claim 32, wherein the nucleic acid-containing specimen is selected from pancreatic ductal epithelium, pancreatic juice, or pancreatic tissue.

39. The method of claim 38, wherein the nucleic acid-containing specimen is pancreatic fluid.

40. The method of claim 32, wherein the pancreatic carcinoma is selected from pancreatic ductal adenocarcinoma, chronic pancreatitis, islet cell tumor, or serus cystadenoma.

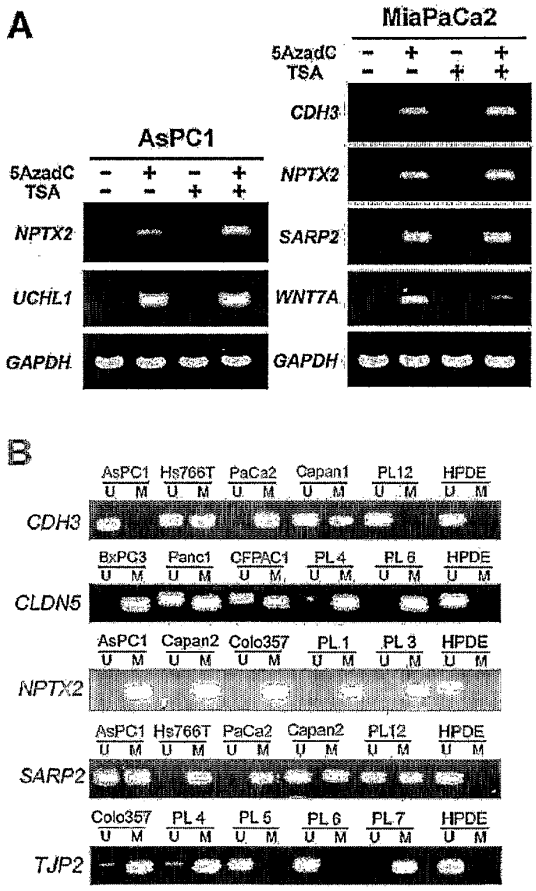

A
AsPC1
5AzadC
TSA
NPTX2
UCHL1
GAPDH
MiaPaCa2
5AzadC
TSA
CDH3
NPTX2
SARP2
WNT7A
GAPDH
B
AsPC1 Hs766T PaCa2 Capan1 PL12 HPDE
CDH3
BxPC3 Panc1 CFPAC1 PL4 PL6 HPDE
CLDN5
AsPC1 Capan2 Colo357 PL1 PL3 HPDE
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AsPC1 Hs766T PaCa2 Capan2 PL12 HPDE
SARP2
Colo357 PL4 PL5 PL6 PL7 HPDE
TJP2

Figure 1

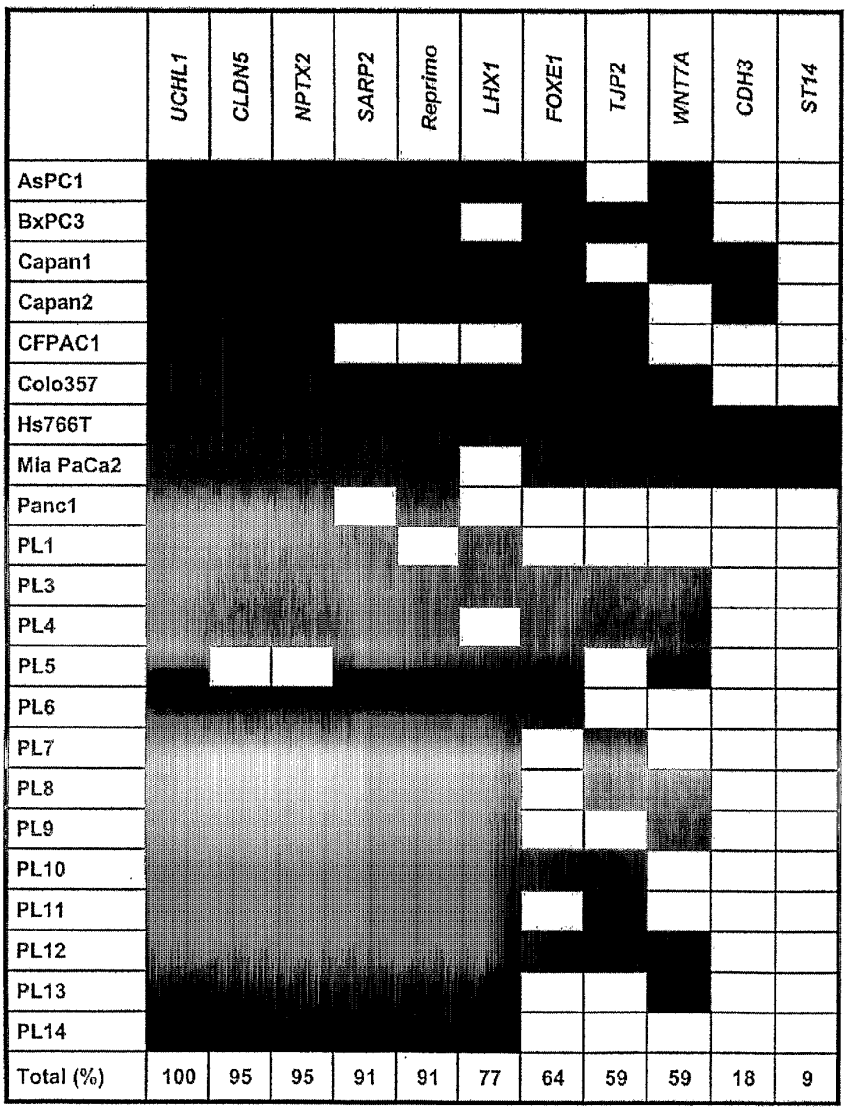

UCHL1
CLDN5
NPTX2
SARP2
Reprimo
LHX1
FOXE1
TJP2
WNTTA
CDH3
ST14
AsPC1
BxPC3
Capan1
Capan2
CFPAC1
Colo357
Hs766T
Mia PaCa2
Panc1
PL1
PL3
PL4
PL5
PL6
PL7
PL8
PL9
PL10
PL11
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PL13
PL14
Total (%)
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91
91
77
64
59
59
18
9

Figure 2

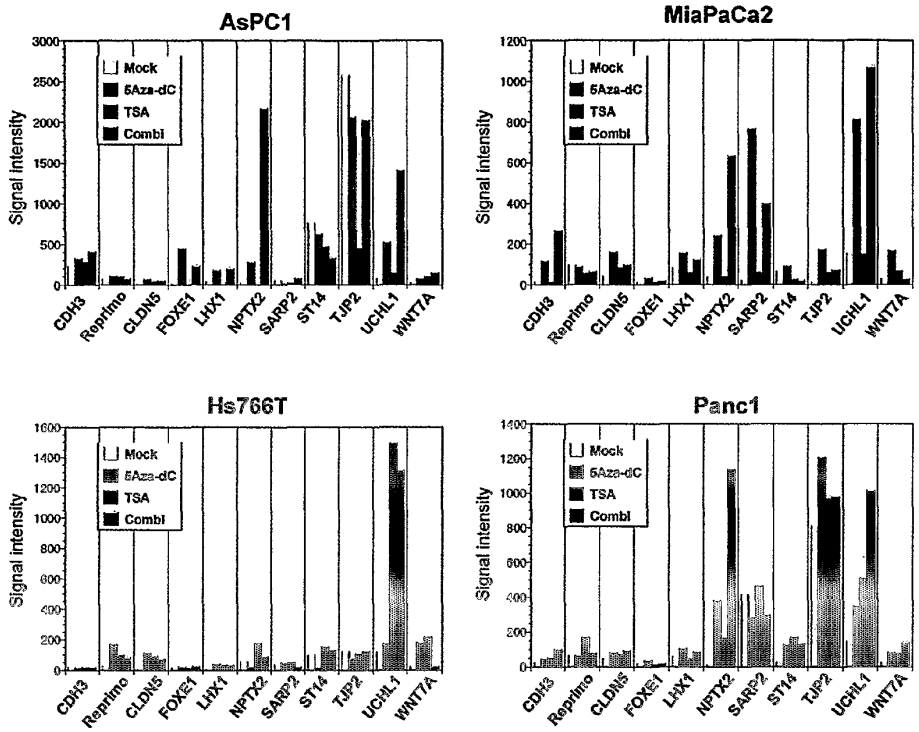

AsPC1
MiaPaCa2
Hs766T
Panc1
Mock
5Aza-dC
TSA
Combi
Signal intensity
CDH3
Reprimo
CLDN5
FOXE1
LHX1
NPTX2
SARP2
ST14
TJP2
UCHL1
WNT7A

Figure 3

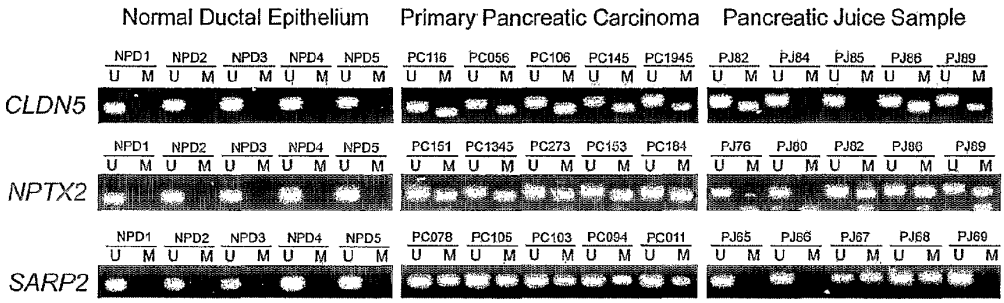

Normal Ductal Epithelium
Primary Pancreatic Carcinoma
Pancreatic Juice Sample
CLDN5
NPTX2
SARP2
NPD1 NPD2 NPD3 NPD4 NPD5
PC116 PC056 PC106 PC145 PC1945
PJ82 PJ84 PJ85 PJ86 PJ89
PC151 PC1345 PC273 PC153 PC184
PJ76 PJ80 PJ82 PJ86 PJ89
PC078 PC105 PC103 PC094 PC011
PJ65 PJ66 PJ67 PJ68 PJ69
U M

Figure 4

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LOGINID:SSSPTAU187BXS

PASSWORD:
TERMINAL (ENTER 1, 2, 3, OR ?):2


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 NEWS  3  APR 30  Derwent Markush Resource (DWPIM) Database Enhancements
 NEWS  4  OCT  7  New CAS FORMULATIONS TM Database Launched on STNext
 NEWS  5  OCT  8  Third 2019 update to Emtree(TM) now available on STN(R)
 NEWS  6  NOV  8  Derwent Markush Resource (DWPIM) Enhanced to Improve Search
                  and Retrieval Quality
 NEWS  7  NOV 15  INPADOC Enhanced with More Current and Complete Japanese Legal
                  Status Information.
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=> File medline biosis
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                       0.18       0.18
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FILE 'BIOSIS' ENTERED AT 14:21:14 ON 14 JAN 2020
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=> t l1

L1   ANSWER 1 OF 1  BIOSIS  COPYRIGHT (c) 2020 Clarivate Analytics  on STN
AN   2008:315116  BIOSIS
DN   PREV200800312994
TI   N-MYC downstream regulated gene 4 (NDRG4) promoter methylation is a
     sensitive and specific biomarker for colorectal cancer.
AU   Melotte, Veerle [Reprint Author]; Lentjes, Marjolein; van den Bosch,
     Sandra; Hellebrekers, Debby; Wouters, Kim; Daenen, Kathleen; Louwagie,
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     Bakker, Carolina; Oort, Frank; Meijer, Gerrit; Jonkers, Daisy; Herman,
     James; de Bruine, Adriaan; van Engeland, Marton
CS   Univ Maastricht, Maastricht, Netherlands
SO   Cellular Oncology, (2008) Vol. 30, No. 2, pp. 181.
     Meeting Info.: Meeting of the International-Society-for-Cellular-Oncology
     (ISCO 2008). Amsterdam, NETHERLANDS. March 05 -08, 2008. Int Soc Cellular
     Oncol.
     ISSN: 1570-5870.
DT   Conference; (Meeting)
     Conference; (Meeting Poster)
LA   English
ED   Entered STN: 21 May 2008
     Last Updated on STN: 21 May 2008


=> FIL STNGUIDE
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                      13.76      13.94

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FILE CONTAINS CURRENT INFORMATION.
LAST RELOADED: Nov 22, 2019 (20191122/UP).

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The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> d his

     (FILE 'HOME' ENTERED AT 14:21:02 ON 14 JAN 2020)

     FILE 'MEDLINE, BIOSIS' ENTERED AT 14:21:14 ON 14 JAN 2020
L1           1 S NDRG4 AND (METHYLATION OR HYPERMETHYLATION) AND PY<2009

     FILE 'STNGUIDE' ENTERED AT 14:26:04 ON 14 JAN 2020

=> loy y
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LOY IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

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COST IN U.S. DOLLARS                            SINCE FILE      TOTAL
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FULL ESTIMATED COST                                 2.34      16.28

STN INTERNATIONAL LOGOFF AT 14:33:38 ON 14 JAN 2020
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	2	(cancer carcinoma) same (PHACTR3 NDRG4 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFRP2 CDO1) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:40
L2	1	"200483399"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:42
L3	0	"200483399A2"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:43
L4	0	"200483399A2"	EPO	OR	OFF	2020/01/15 11:11
L5	0	"200483399"	EPO	OR	OFF	2020/01/15 11:11
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;	OR	OFF	2019/06/23 13:34

			DERWENT; IBM_TDB			
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
S8	0	NDGR4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S9	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S10	6	NDRG4 same epigenetic and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:58
S11	24	"2006113770"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:02
S12	4	"2006113770" and NDRG4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:11
S13	6	NDRG4 same (epigenetic methylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S14	6	NDRG4 same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S15	0	"N-myc downstream regulated gene 4" same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	OFF	2020/01/14 14:24

			DERWENT; IBM_TDB			
S16	0	"N-myc downstream regulated gene 4" and (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S17	0	"N-myc downstream regulated gene 4" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:25
S18	1	"20060019256" and uptg	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:55
S19	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 15:35
S20	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:07
S21	3	fecal same (antibody antibodies immunoassay) same (DNA RNA polynucleotide "nucleic acid" gene) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:26

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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 NON-FINAL OFFICE ACTION MAILED 01/21/2020

EFS WEB FILED
Commissioner for Patents
P.O. Box 1450
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CERTIFICATE OF EFS WEB TRANSMISSION UNDER 37 C.F.R. § 1.8

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Dated: May 21, 2020 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This paper is responsive to the Office Action mailed 01/21/2020, with response due by 04/21/2020. A one-month extension of time, to 05/21/2020, is requested. 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 a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Remarks begin on Page 2.

REMARKS

Claims 82-84 and 87-100 are pending and under examination. In the Office Action mailed 01/21/2020 ("Office Action"), the Examiner made the following rejections:

I. Claims 82-84 and 87-100 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Applicant admissions in view of US 2008/0227208 A1 ("Yee"), US 5,198,365 ("Grow"), and US 2008/0124714 A1 ("Shuber").

II. Claim 99 stands rejected as allegedly being unpatentable over Applicant admissions in view of Yee, Grow, Shuber, further in view of WO 2004/083399 A2 ("Goggins").

The claims are not obvious

I. Claims 82-84 and 87-100 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Applicant admissions in view of Yee, Grow, and Shuber.

The Examiner points to Applicant's Specification as allegedly teaching an extensive list of features, including:

- That immunochemical assays for detecting human hemoglobin in fecal sample were known in the art (Office Action, ¶17);
- That detection of colorectal carcinoma based upon determining methylation of a number of different genes is disclosed in is disclosed in WO 2008/084219 Al (Van England et al.) (Office Action, ¶18);
- That methods for isolating DNA are known in the art. (Office Action, ¶19);
- That kits for amplifying DNA in stool samples are commercially available (Office Action, ¶20);
- That kits for isolating DNA from fecal samples are commercially available. (Office Action, ¶21);
- 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. (Office Action, ¶22);

- That methods for performing **MSP,** wherein one employs primer pairs designed to distinguish methylated DNA from unmethylated **DNA,** was known in the art. (Office Action, ¶23); and
- A variety of nucleic acid amplification techniques (Office Action, ¶25).

The Examiner points to Yee as teaching a 10 ng/ml of hemoglobin as a threshold value, to Grow as teaching freezing a portion of a fecal sample and preparation of a fecal sample for immunoassay, and to Shuber as teaching treating a stool sample with a nucleic acid stabilizing solution for nucleic acid analysis.

Without acquiescing to the Examiner's characterization of the teachings of Yee, Grow, and Shuber, Applicant points out that none of Yee, Grow, or Shuber teach or suggest analysis of a single collected fecal sample for both 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 and alleged admissions 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.

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 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 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 gene/sequence of interest." (Office Action, ¶35). 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, ¶36). The Examiner provides no additional analysis or argument in support of making the combination.

Examiner has not provided the "articulated reasoning" required 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 is no more than a conclusory statement and is not sufficient to establish prima facie obviousness of the instant claims 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 of 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 the use of one testing method alone. Rather, the skilled person considering the cited art could only guess at whether expending the time and effort to conduct both blood/hemoglobin testing and nucleic acid testing on a fecal sample *might* produce some beneficial result, rather than merely producing redundant information.

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 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, and has further failed to establish that the skilled person would have a reasonable expectation success. Applicant therefore respectfully requests that the rejection of claims 82-84 and 87-100 be withdrawn.

II. Claim 99 stands rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Applicant admissions in view of Yee, Grow, and Shuber as applied to claims 82-84, 87-98, and 100 above, and further in view of Goggins.

The combination of Yee, Grow, and Shuber as applied to claims 82-84, 87-98, and 100 is discussed above. The Examiner admits that the combination of Yee, Grow, Shuber, and Applicant's alleged admissions does not teach the genes listed in claim 99. (Office Action, page 13). The Examiner points to Goggins as teaching, inter alia, the FOXE1 mutation in association with pancreatic carcinoma (Office Action, page 14). Applicant disagrees that Goggins, pointing to the FOXE1 marker as being associated with pancreatic cancer, would point the skilled person to process a fecal sample in manner of the instant claims. Nothing in Goggins suggests any utility in testing fecal samples using both blood/hemoglobin testing *and* nucleic acid testing for

the FOXE1 gene for pancreatic cancer, and Goggins' focus on FOXE1 as a useful marker for pancreatic carcinoma does not in any way suggest a utility in line with the "intense interest in detection of colorectal carcinoma in an individual," the sole motivation offered by the Examiner for combining Yee, Grow, and Shuber. Rather, if, as the Examiner has argued, detection of methylation status of the FOXE1 gene in a sample collected from a colon is indicative of *pancreatic* carcinoma (see, *e.g.,* Goggins, claim 17), then the skilled person considering the teachings of Goggins would look to markers other than FOXE1 for detecting *colorectal* carcinoma by analysis of such samples.

Furthermore, Goggins fails to cure the deficiencies of the Examiner's arguments regarding Yee, Grow, and Shuber set forth above and, for the same reasons, these arguments fail to establish the obviousness of claim 99 in view of the combination of Yee, Grow, Shuber and Goggins. Applicant therefore respectfully requests that the rejection of claim 99 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: May 21, 2020 /Mary Ann D. Brow /

Mary Ann D. Brow
Registration No. 42,363

CASIMIR JONES, S.C.
2275 Deming Way, Suite 310
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Electronic Patent Application Fee Transmittal

Application Number:	15634607
Filing Date:	27-Jun-2017
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Filer:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239.304

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:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**200**

Electronic Acknowledgement Receipt	
EFS ID:	39513398
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
Filer Authorized By:	
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	22-MAY-2020
Filing Date:	27-JUN-2017
Time Stamp:	00:16:50
Application Type:	Utility under 35 USC 111(a)

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Payment Type	CARD
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RAM confirmation Number	E20205L117196854
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	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Applicant Arguments/Remarks Made in an Amendment		2	6	
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30740 c0cab143aa6c76a3584398566da62c6fde4ec368	no	2
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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	LOUWAGIE
				Art Unit	1634
				Examiner Name	SISSON
Sheet	1	of	2	*Attorney Docket Number*	EXCTD-35239.304

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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		KIM et al., Noninvasive Molecular Biomarkers for the Detection of Colorectal Cancer. BMB Rep. 2008 Oct 31;41(10):685-92	
		TURGEON et al., Fecal DNA-Based Detection of Colorectal Neoplasia. Curr Colorectal Cancer Rep. 2007 Oct;3(4):171-177	

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				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	LOUWAGIE
				Art Unit	1634
				Examiner Name	SISSON
Sheet	2	of	2	*Attorney Docket Number*	EXCTD-35239.304

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☐ 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).

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☐ 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.

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Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2020-07-07
Name/Print	Mary Ann D. Brow	Registration Number	42363

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BMB
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Mini Review

Noninvasive molecular biomarkers for the detection of colorectal cancer

Hye-Jung Kim[1], *Myeong-Hee Yu*[2], *Hoguen Kim*[3], *Jonghoe Byun*[4] *& Cheolju Lee*[1,*]

[1]Life Sciences Division and [2]Functional Proteomics Center, Korea Institute of Science and Technology, Seoul 136-791, Korea, [3]Department of Pathology, Yonsei University College of Medicine, Seoul 120-752, Korea, [4]Department of Molecular Biology, Dankook University, Yongin-si, Gyeonggi-do 448-701, Korea

Colorectal cancer (CRC) is the third most common malignancy in the world. Because CRC develops slowly from removable precancerous lesions, detection of the disease at an early stage during regular health examinations can reduce both the incidence and mortality of the disease. Although sigmoidoscopy offers significant improvements in the detection rate of CRC, its diagnostic value is limited by its high costs and inconvenience. Therefore, there is a compelling need for the identification of noninvasive biomarkers that can enable earlier detection of CRC. Accordingly, many validation studies have been conducted to evaluate genetic, epigenetic or protein markers that can be detected in the stool or in serum. Currently, the fecal-occult blood test is the most widely used method of screening for CRC. However, advances in genomics and proteomics combined with developments in other relevant fields will lead to the discovery of novel non invasive biomarkers whose usefulness will be tested in larger validation studies. Here, noninvasive molecular biomarkers that are currently used in clinical settings and have the potential for use as CRC biomarkers are discussed. [BMB reports 2008; 41(10): 685-692]

Introduction

Colorectal cancer (CRC) is the third most common malignancy in the world. In addition, there are approximately 1,000,000 new cases of CRC and 500,000 deaths associated with CRC each year. Indeed, CRC represents one of the primary causes of cancer deaths in Europe and the USA (1). In Korea, CRC is the fourth leading cause of mortality by cancer, and its incidence is increasing (2). CRC is believed to develop slowly via a progressive accumulation of genetic mutations; therefore, the risk of recurrence and subsequent death due to CRC is closely related to the stage of the disease at the time of primary diagnosis. Recent studies have shown that shifting the detection of the disease to an earlier stage via mass screening and intervening at this stage can reduce the risk of death from CRC (3, 4). These findings strongly demonstrate the clinical need for biomarkers for early detection of CRC.

Biomarkers are substances that are used as indicators of a biological state. Accordingly, biomarkers have characteristics that enable them to be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (5). One of the key requirements of a test for CRC is that it must allow detection of the disease at earlier stages so that the disease can be cured effectively. Accordingly, such tests should have high sensitivity and specificity while producing a low number of false-negative and false-positive results to prevent subjecting healthy individuals to unnecessary colonoscopies. Sigmoidoscopy offers significant improvements in detection rates for CRC. However, the diagnostic value of sigmoidoscopy is limited with regards to costs, risks, and inconvenience (6, 7). Noninvasive biomarkers can be analyzed relatively easily and economically; therefore, they have the potential to greatly enhance screening acceptance. Several noninvasive tests for CRC detection are available, of which the fecal-occult blooding test (FOBT) is the most commonly used (8-10). However, this method lacks sensitivity as well as specificity for screening an average risk population. As a result, new cancer biomarkers that will further enhance the detection of the disease and trigger follow-up colonoscopies when necessary must be developed. In addition to such screening biomarkers, prognostic markers that predict the likely course of the cancer, stratification markers that predict the likely response to a drug prior to beginning treatment, and efficacy markers that monitor the efficacy of drug treatment may eventually reduce the mortality rate of CRC.

Recent advances in genomics and proteomics have contributed to our understanding of pathways that control the growth, differentiation, and death of cells. Genomic techniques such as DNA microarray analysis and proteomic methods such as 2-dimensional electrophoresis and mass spectrometry are now commonly used to evaluate the expression profiles of genes and proteins in cells, tissues, and bodily fluids (11, 12).

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Identification of genes or proteins that are characteristic of the development of cancer can potentially uncover biomarkers that will aid in the diagnosis of CRCs. This review will focus on potential biomarkers that have recently been discovered and may be used in the future to detect CRC using non-invasively-collected samples as well as biomarkers that are currently being used in clinical settings (Table 1).

Fecal markers

Fecal hemoglobin

Stool-based screening for CRC is simple, inexpensive and the least invasive method of screening available (13). FOBT, which is the most widely used screening modality for CRC, detects hemoglobin enzymatically or immunologically (14). Enzymatic FOBT measures the peroxidase-like activity of hemoglobin that originates from any source; therefore, it is susceptible to both colorectal and upper gastrointestinal bleeding. In addition, the ingestion of certain foods (red meats, fruits and vegetables) and medicines (non-steroidal anti-inflammatory drugs) can also yield false-positive results. Immunological FOBT uses antibodies that specifically detect human hemoglobin; therefore, it is not impacted by plant peroxidase in the diet. An important limitation of the FOBT is its relatively poor sensitivity at detecting early-stage lesions. In addition, FOBT shows low sensitivity for the detection of both adenomas (~10%) and CRCs (40-85%). Finally, large randomized clinical trials have shown that FOBT is not very reliable and that they reduce cancer mortality by only 30% (3, 15).

Genes and epigenetic markers

Colonocytes, which are shed into the fecal stream, provide informative material that can be used to detect genes and epigenetic markers in feces (16). Unlike fecal blood, which is shed only intermittently, it is believed that colonocytes are shed continuously. Furthermore, the shedding of colonocytes from CRC occurs more frequently than from normal colonic epithelium. Fecal colonocytes are assessed by analyzing DNA mutations for targets such as K-*ras*, *p53*, and adenomatous polyposis coli (*APC*), by analyzing epigenetic markers such as microsatellite instability (MSI), or by measuring unfragmented long-form DNA (L-DNA).

K-*ras*, which encodes a Ras family protein, functions as a guanine nucleotide binding protein that is involved in a signal transduction pathway that includes the phosphatidylinositol-3-kinase and serine/threonine protein kinase B pathways (17). K-*ras* mutations have been found in 40-50% of sporadic colon cancers and adenomas (18). In addition, studies have demonstrated that K-*ras* mutations are present in aberrant crypt foci (a suspected pre-cancerous lesion) in 13-95% of all CRC cases (19-21), which suggests that K-*ras* mutations may be an important early event in tumorogenesis.

p53 encodes a tumor suppressor protein that regulates the expression of genes involved in apoptosis, angiogenesis, the cell cycle and maintenance of the genome (22). Approximately 50% of all human cancers contain mutated *p53* genes, and 30-60% of

Table 1. Noninvasive molecular biomarkers for the detection of CRC

Analyte	Subject	Type	Status
Fecal hemoglobin	Stool	Protein	IU
K-*ras*	Stool	DNA	CV
APC	Stool	DNA	CV
L-DNA	Stool	DNA	CV
p53	Stool	DNA	CV
CEA	Serum	Protein	IU
CA19.9	Serum	Carbohydrate	IU
TIMP-1	Serum	Protein	CV
Spondin-2, DcR3, Trail-R2, Reg IV, MIC 1	Serum	Protein	PD
PSME3	Serum	Protein	PD
NNMT	Serum	Protein	PD
CRMP-2	Serum	Protein	PD
SELDI (apolipoprotein C1, C3a-desArg, alpha1-antitrypsin, transferrin)	Serum	Protein	PD
HNF 1-3	Serum	Protein	PD
MIF	Serum	Protein	PD
M-CSF	Serum	Protein	PD
M2-PK	Serum	Protein	PD
Prolactin	Serum	Protein	PD
Septin 9	Plasma	DNA	PD
Five-gene panel (*CDA, BANK1, BCNP1, MS4A1, MGC20553*)	WBC	DNA	PD
CCSA-2, -3 -4	Serum	Protein	PD
MMP-9, -7	Serum	Protein	PD
Laminin	Serum	Protein	PD

IU, in use; CV, clinical validation; PD, preclinical development

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CRCs have demonstrated mutations in the gene that vary based on the stage, grade and location of the cancer (23) These mutations appear to be formed relatively late in the genesis of CRC, and altered *p53* has only a modest impact on outcome. Therefore, the relatively low mutation rate of *p53* in early-stage tumors limits its sole use in DNA-based detection of CRC.

APC protein is another tumor suppressor that assembles on a scaffold protein, axin, with β-catenin and glycogen synthase kinase 3β to coordinate the regulation of β-catenin signaling (24). Inactivation of the APC protein is responsible for both inherited and sporadic forms of CRC. Like K-*ras*, mutation of *APC* appears to be an early-genetic event in the progression from adenoma to carcinoma, which indicates its potential for use as a screening marker. However, unlike K-*ras*, the mutations are distributed throughout the coding region, thereby making it technically difficult and time consuming to detect all of the potential mutations during screening programs for CRC (21, 25, 26).

Microsatellites are stretches of short DNA sequences that contain a motif of 1-5 nucleotides that are tandemly repeated (27). The most common microsatellite in human DNA is a dinucleotide repeat of cytosine and adenine. These tandem repeats occur throughout the human genome. MSI occurs when microsatellites undergo a change in length that covers approximately 15% of the CRC. MSI tumors show a better prognosis than stage-matched microsatellite stable tumors (28). In sporadic CRCs, the development of MSI most commonly occurs due to epigenetic silencing of the DNA mismatch repair gene, *MLH1* (29). There are several MSI markers, among which *BAT26* is probably the most widely used.

Shedding of colonocytes is a normal consequence of exfoliation (30). Untransformed colonocytes are shed continuously from the colonic mucosa. These cells usually undergo apoptosis, which is characterized by DNA fragmentation and laddering. Conversely, malignant colonocytes shed from CRC tumors have a decreased rate of apoptosis relative to normal colonocytes, which facilitates detection of intact genomic DNA (L-DNA) as a potential stool-based marker. Boynton *et al.* (31) amplified six genomic fragments of different length from each of four different genetic loci (*APC, p53, BRCA1, and BRCA2*) using fecal specimens collected from 25 CRC patients and 77 controls. In their study, when a positive L-DNA was defined as >18 bands detected from a possible 24 bands (4 loci × 6 fragments), the specificity for CRC detection was 97% and the sensitivity was 57%.

A large population-based study conducted by Imperiale *et al.* revealed that a fecal DNA panel consisting of 21 mutations (3 in the K-*ras* gene, 10 in the *APC* gene, and 8 in the *p53* gene; the MSI marker BAT-26; and L-DNA) detects a greater proportion of important colorectal neoplasia than FOBT without compromising specificity (32). In addition, the sensitivity of the fecal DNA panel was 52% for invasive caners and 41% for invasive cancers plus adenomas with high-grade dysplasia, whereas that of the FOBT was 13% for the former and 14% for the latter. In subjects with negative findings on colonoscopy, the DNA panel had a specificity of 94%, whereas FOBT had a specificity of 95%. Taken together, the results of this study clearly demonstrate that the DNA panel has a higher sensitivity than FOBT without reduced specificity.

Serum or blood markers

CEA

Carcinoembryonic antigen (CEA) is a high molecular weight glycoprotein that belongs to the immunoglobulin superfamily. The carboxy-terminal of CEA contains a hydrophobic region that is modified to provide a glycosyl phosphatidylinositol link to the cell membrane. Although its presence can be determined in biopsy samples, it is usually identified in serum. This protein has been used for many years as a biomarker of CRCs as well as for other cancers (33). Specifically, high CEA levels are associated with cancer progression, and levels of the marker are expected to fall following cancer surgery (34). However, in the absence of cancer, high CEA levels may also occur in response to other conditions such as hepatitis, inflammatory bowel disease, pancreatitis, and obstructive pulmonary disease. Furthermore, CEA may not be detected when cancer is in advanced stages. As a result, CEA does not provide sufficient sensitivity and reliability for the early detection of cancer. Indeed, using a cut-off point of 2.5 ng/ml results in the sensitivity of CEA for early CRC (i.e., Dukes' A and B disease) being only 30-40% with a specificity of 87% (9). Therefore, the NIH does not recommend that CEA be used to screen for early CRC (33). Instead, the potential value of the CEA test lies in its use to measure the course of the progression of cancer as a prognostic marker once it has been diagnosed, with higher CEA levels being indicative of greater disease severity and a poorer prognosis.

CA 19-9

Carbohydrate antigen 19-9 (CA 19-9), which is the second most investigated gastrointestinal tumor marker, is known to be a sialylated Lewis-a antigen (35). CA 19-9 was originally defined by a monoclonal antibody produced by a hybridoma prepared from the spleen cells of a mouse that had been immunized with the human colorectal carcinoma cell line, SW 1116. Although CA 19-9 is the best marker available for pancreatic adenocarcinoma, it is less sensitive than CEA for CRC and provides less information than CEA when used for monitoring patients that have already been diagnosed with CRC (36). Other carbohydrate antigens such as CA 50, CA 195, CA 242, CA M26, CA M25, CA M43 and CA 72-4 have also been evaluated extensively (37); however, due to their observed sensitivity, stage dependency and specificity, these antigens are not useful markers for the detection of CRC.

Tissue inhibitor of metalloproteinase type 1

Tissue inhibitor of metalloproteinase type 1 (TIMP-1) is a mul-

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tifunctional glycoprotein that inhibits most matrix metalloproteinases (MMPs). The total levels of TIMP-1 have been shown to be significantly higher in patients with both colonic and rectal cancers, while healthy blood donors have a low and very narrow range of plasma TIMP-1 levels (38, 39). More importantly, TIMP-1 is capable of detecting early stage colon cancer with a sensitivity and specificity comparable to those obtained when detecting late stage cancers. Conversely, patients with colon adenomas, inflammatory bowl diseases or primary breast cancer do not show increased plasma levels of total TIMP-1 (40). Preoperative TIMP-1 levels have been proposed as a stage-independent prognostic marker for CRC in multivariate statistical analyses in two separate high-powered studies (41, 42). However, the results of these studies revealed that elevated levels of TIMP-1 were not restricted to advanced stages of CRC alone; therefore, larger studies are required to validate the use of TIMP-1 for early diagnosis and evaluation of the prognosis of CRC.

Five- serum-marker panel (spondin-2, DcR3, Trail-R2, Reg IV, MIC 1)

diaDexus Inc. recently evaluated four serum biomarkers, spondin-2, tumor necrosis factor receptor superfamily member 6B (DcR3), TRAIL receptor 2 (TRAIL-R2) and Reg IV in 600 serum samples in collaboration with the Mayo Clinic (43). All four markers, as well as a fifth marker, macrophage inhibitory cytokine 1 (MIC1), were found to be elevated in patients with CRC when compared to normal controls and patients with benign disease. In addition, this five-serum marker panel offers better sensitivity and specificity than CEA. Accordingly, the company is currently developing biomarker panels that include the 5 markers as diagnostic modalities to improve the detection rate of early stage CRC.

Nicotinamide N-methyltransferase and proteasome activator complex subunit 3

Roche Diagnostics GmbH utilized two-dimensional gel electrophoresis and mass spectrometry to analyze 16 matched CRC and adjacent normal tissue samples. Proteins found to be elevated in cancer tissue were then further validated with serum samples. Elevated levels of nicotinamide N-methyltransferase (NNMT) and proteasome activator complex subunit 3 (PSME3), which are not predicted to be secreted, were found in serum from patients with CRC (44, 45). Validation studies using 109 CRC samples, 317 healthy control samples, and 87 samples from patients with benign bowel diseases revealed that the diagnostic accuracy of PSME3 was similar to that of CEA, and that NNMT was better than CEA at detecting CRC. However, the abundance of PSME3 is less stage-dependent than CEA.

Collapsin response mediator protein-2

Although direct analysis of human samples may sometimes be very challenging, discovering candidate biomarkers from cancer cell lines and subsequent validation in human samples is plausible (46, 47). By analyzing the secretomes of 21 cancer cell lines derived from 12 cancer types, Wu *et al.* identified collapsin response mediator protein-2 (CRMP-2) and evaluated it as a potential CRC biomarker in the serums of 201 CRC patients and 210 healthy controls (48). The use of CRMP-2 alone showed better sensitivity but poorer specificity than CEA. However, combined detection using CEA and CRMP-2 produced better sensitivity (77%) and specificity (95%) than detection using either of these markers alone (43 and 61% sensitivity, respectively; 87 and 65% specificity, respectively). Therefore, CRMP-2 may be a valuable serum marker when used in combination with CEA.

Six SELDI peaks corresponding to apolipoprotein C1, complement C3a-desArg, α1-antitrypsin and transferrin

Surface-enhanced laser desorption/ionization (SELDI) mass spectrometry is a technology that can produce proteomic fingerprints from biological samples using a relatively high-throughput platform (49). In this method, samples are applied to proteinchip arrays coated with chromatographic surfaces and then analyzed by mass spectrometry. Martin *et al.* used SELDI to investigate the serum proteome of 62 CRC patients and 31 noncancer subjects (50). They identified four proteins with diagnostic potential: apolipoprotein C1, complement C3a-desArg (the stable form of C3a anaphylatoxin), α1-antitrypsin and transferrin. Artificial neural networks trained using only the intensities of six SELDI peaks corresponding to the identified proteins were able to classify the patients enrolled in the study with 95% sensitivity and 91% specificity, while the use of CEA to evaluate the same sample set showed 54% sensitivity and 93% specificity.

Habermann *et al.* demonstrated that C3a-desArg is present at significantly higher levels in serum from patients with colorectal adenomas and carcinomas than in serum from healthy individuals (51). Specifically, in a blinded validation study (n = 59), the use of C3a-desArg alone predicted the presence of colorectal malignancy with a sensitivity of 97% and a specificity of 96%. C3a-desArg and other proteins corresponding to SELDI peaks are common serum proteins, and changes in their concentrations most likely reflect epiphenomena rather than secretion by cancer cells.

α-Defensins

Two independent approaches using SELDI-TOF to analyze the protein profiles of colon cancer serum and protein profiles of CRC tumors demonstrated that human neutrophil peptides (HNP)-1, HNP-2 and HNP-3, also known as α-defensin-1, α-defensin-2, and α-defensin-3, are up-regulated in patients with CRC (52, 53). Indeed, the HNP1-3 level in the serum of 48 CRC patients and 42 normal controls was capable of identifying CRC with a sensitivity of 69% and a specificity of 100%. However, a larger study is required to refine and validate the diagnostic accuracy of these findings.

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Macrophage migration inhibitory factor

Based on an observation that the gene expression level of macrophage migration inhibitory factor (MIF) is elevated in CRC tissues, our group has evaluated the use of the protein as a potential biomarker for CRC. In an analysis of serum samples of 129 patients with colon cancer and 53 healthy control subjects, the serum MIF level was found to be significantly increased in patients with CRC (54). Although the specificity of MIF is not as high as that of CEA (90.6% vs. 100.0%), MIF is more sensitive during early cancer detection (47.3% vs. 29.5%), which suggests that MIF may be used as a diagnostic marker in CRC.

Macrophage-colony stimulating factor

The serum levels of both macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor are significantly higher in CRC patients than in healthy subjects (55, 56). In addition, serum levels of M-CSF are more associated with lymph node metastasis than CEA and CA 19-9, which suggests that serum M-CSF elevation in CRC patients might help predict the risk of lymph node metastasis of this tumor. Finally, M-CSF appears to offer additional information to that presented by classic prognostic factors.

Prolactin

Prolactin, which is a hormone with multiple biological actions that is synthesized by the anterior pituitary gland, is elevated in patients with CRC. A study that evaluated 47 CRC patients and 51 healthy controls revealed that prolactin can predict CRC with a sensitivity and specificity of 77% and 98%, respectively (57).

M2-pyruvate kinase

M2-pyruvate kinase is an isoform of glycolytic enzyme pyruvate kinase. Although the protein is a cytosolic enzyme, it is liberated into circulation via a mechanism that is not yet known. However, it has been suggested that M2-pyruvate kinase is released into circulation from dying cancer cells. Therefore, M2-pyruvate may be a useful marker for the detection of CRC. In addition, two independent studies revealed that the use of M2-pyruvate kinase for the detection of CRC has a sensitivity of 48-58% and a specificity of 90-95%. Furthermore, when combined with CEA, the sensitivity of M2-pyruvate increases without decreasing the specificity (58, 59).

Methylated septin-9 DNA

The assessment of epigenetic events is one of the most promising means of identifying biomarker candidates for the early detection of cancer. DNA methylation in which cytosines within the palindromic dinucleotide 5'-CpG-3' sequence are methylated shapes the chromatin structure of DNA according to its functional state (60). The cancer genome is frequently characterized by hypermethylation of specific genes. Therefore, Epigenomics AG has developed a blood-test for CRC that is based on methylation of *SEPT9*, *NGFR* and *TMEEF2* (61). To evaluate this test, free-floating DNA was extracted from plasma samples of 133 CRC patients and 179 healthy controls in the same age range, and the methylation levels were then measured using restriction enzyme-based qPCR. The biomarker with the highest performance was found to be *SEPT9*, which was capable of detecting CRC with a specificity and sensitivity of 95% and 52%, respectively, when a cutoff of 0.011 μg/L of methylated *SEPT9* DNA was used.

Five gene markers in whole blood

Gene expression patterns in the peripheral blood reflect changes that occur within the cells and tissues of the body (62). Han *et al.* extracted total RNA from the white blood cells of peripheral blood and identified differentially regulated genes using a microarray (63). Specifically, they used a panel comprised of five genes including B-cell scaffold protein with ankyrin repeats 1 (BANK1), B-cell novel protein 1 (*BCNP1*), cytidine deaminase (*CDS*), FERM domain containing 3 (*MGC20553*), and membrane-spanning 4-domains, subfamily A, member 1 (*MS4A1*) to detect CRC and found that this test had a sensitivity of 88-94% and a specificity of 64-77%.

Other markers

Three proteins, colon cancer-specific antigen (CCSA)-2, CCSA-3 and CCSA-4, have shown promise as markers for the detection of CRC. Getzenberg *et al.* identified several nuclear matrix proteins from CRC cell lines and tested some of them to determine if they would be useful as cancer biomarkers (64). Using a cutoff value of 2 μg/ml for CCSA-3, both CRC and advanced adenoma were detected with 89% sensitivity and 82% specificity. In addition, the sensitivity and specificity was 85% and 91%, respectively, when CCSA-4 was used with a cutoff value of 0.3 μg/ml. Finally, the use of CCSA-2 at a cutoff of 10.8 μg/ml had an overall specificity of 78% and sensitivity of 97% when used to separate individuals with advanced adenomas and CRC from normal, hyperplastic, and nonadvanced adenoma populations (65). Although the initial studies have had promising outcomes, the molecular identities of the three proteins have not yet been publically disclosed.

Remodeling of the extracellular matrix is important in the development of cancers, and several extracellular matrix proteins that can be liberated into circulation have been evaluated as potential biomarkers. The results of these evaluations have revealed that the serum levels of MMP9 and MMP7 depend on the presence of colorectal malignancy (66, 67). In addition, it has been suggested that serum laminin and MMP7 can be used as independent prognostic markers of CRC (67, 68).

Conclusion and perspectives

FOBT is currently the only screening modality for CRC. DNA-based fecal markers are promising but are not widely

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used in clinical settings. In addition, a lack of sensitivity and specificity preclude the use of all existing serum markers for the early detection of CRC. CEA is used to monitor therapy in advanced CRC, and the preoperative level of CEA is used to provide prognostic information. However, there is insufficient evidence for routine use of other classic serum markers such as carbohydrate antigens and TIMP-1. Therefore, large-scale validation studies are required to evaluate the potential for the use of biomarkers that have recently been discovered through omics technology.

Genomics, proteomics, and a combination of both of these methods play major roles in the discovery of biomarkers. As with many other methods, each approach has its advantages and disadvantages. However, in light of the fact that many important functions of proteins require post-translational modification or their interaction with other protein(s), proteomics will continue to play a dominant role in the biomarker field. Clinical proteomics as a principal tool for the detection of differentially expressed proteins in samples from CRC patients versus healthy donors is required to significantly increase the repertoire of candidate biomarkers. In addition, steps should be taken to design adequate clinical trials and build a business model that can be used for the commercialization of newly discovered biomarkers. These efforts will hasten the translation of proteomic discoveries into clinical practice in the not-so-distant future.

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Fecal DNA–Based Detection of Colorectal Neoplasia

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Abstract

Human DNA fragments, as well a as whole colonocytes, can be found in stool and interrogated for mutations associated with carcinogenesis. Multiplexed panels of point mutations in fecal DNA improved the sensitivity of cancer detection (51.6%; 95% CI, 34.8%–68%) compared with fecal occult blood testing (12.9%; 95% CI, 5.1–28.9). However, the multiplex panels are not more sensitive than fecal occult blood testing in detecting advanced adenomas. Markers of epigenetic events, such as DNA methylation of gene promoters in genes not previously known to be associated with carcinogenesis or colorectal cancer, have recently been shown to be potential markers for early detection. Future approaches to improve sensitivity of fecal DNA detection of colorectal adenocarcinoma may require the inclusion of epigenetic biomarkers, the use of colonocytes isolated from feces, or both.

Introduction

Colorectal cancer remains the second leading cause of cancer deaths in the United States and the leading cause in nonsmokers [1]. Although colorectal cancer is preventable, screening rates are lower than those for common solid tumors with established effective screening and early detection tests (breast, cervical, or prostate cancer) [2,3]. When not prevented, colorectal cancer is curable if identified at an early stage. Yet it is estimated that in 2007 only about 50% of eligible persons in the United States will undergo any screening evaluation for colon cancer and fewer than 20% will undergo colonoscopy.

Current colorectal cancer screening recommendations of both the American Cancer Society and the US Preventive Health Services Task Force (USPHSTF) include a "menu of options" for those aged 50 years or older to choose from: yearly fecal occult blood testing (FOBT), flexible sigmoidoscopy every 5 years, a combination of the first two options, colonoscopy every 10 years, or barium enema every 5 years [4,5]. The USPHSTF endorses colonoscopy as the most sensitive and specific test for colorectal cancer screening but says it is "not certain whether the potential added benefits of colonoscopy relative to the screening alternatives are large enough to justify added risks and inconvenience for all patients." Colonoscopy is the final common pathway that provides the benefit for all these menu options. Colonoscopy has been proposed as first-line screening for colorectal cancer by the American Gastroenterology Association (AGA) and the American Society for Gastrointestinal Endoscopy (ASGE). If the US population fully adhered to the current

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screening guidelines, however, the cost, burden, and morbidity would outstrip the health care system's ability to meet the needs [6•].

The currently recommended noninvasive colorectal cancer screening test (FOBT) is limited by several issues:

1. A high false-positive rate, which results in large numbers of unneeded follow-up tests (colonoscopy)
2. A substantial false-negative rate, which results in undetected disease
3. A tendency for the specificity (true negatives divided by true negatives and false positives) to be skewed because the large number of negative subjects without disease (true negatives) overwhelms the relatively high false-positive rate in an environment of low cancer prevalence
4. Low sensitivity (true positives divided by true positives and false negatives) because the true positives are few and even a low rate of false negatives will degrade the sensitivity.

Flexible sigmoidoscopy is minimally invasive but has not been well accepted or utilized. Flexible sigmoidoscopy has an impressive level of performance for detection of colorectal cancers in the distal colon (sensitivity 97% and specificity 94%) [7]. It is credited with 50% to 95% reduction in mortality from left-sided colorectal cancers, and this reduction in mortality may show benefit for up to 16 years. Unfortunately it screens only about one third of the tissue at risk and can miss up to 48% of advanced proximal lesions [8].

Unlike flexible sigmoidoscopy, colonoscopy screens all of the tissue at risk for visible lesions. It has a sensitivity of 97% and a specificity of 98% for the detection of colorectal cancers and a specificity of 90% for adenomas larger than 1 cm [7,9•]. It fails as an ideal first-line screening test because of its poor public acceptance, risk of complications, high cost, and technical difficulty [9•,10•]. Barium enema, although able to screen all the tissue at risk, has a lower sensitivity than colonoscopy, especially for adenomas. Like colonoscopy, it suffers from poor public acceptance as a first-line screening tool [11].

The weaknesses of noninvasive methods in current colorectal screening, combined with adherence and cost limitations of invasive screening procedures, provide a strong rationale for the development of early colorectal cancer detection biomarkers that are simple and cost-effective. Such biomarkers should be able to detect both preinvasive and invasive neoplasms with high sensitivity (low false-negative rate) and modest specificity (an acceptably high false-positive rate). A successful biomarker panel coupled with colonoscopic follow-up only for patients with positive results would reduce the burden and morbidity of screening colonoscopy and most likely would enhance adherence to colorectal screening.

Rationale for Stool-Based Biomarkers for Detection of Colorectal Cancer

Molecular diagnostic tests performed on fecal samples are emerging as potentially important new approaches that may provide accurate and cost-effective early detection of colonic neoplasia. Details of many of the currently employed and novel approaches have been

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recently reviewed [12•]. The concept of fecal screening is to exploit the regional advantage of sampling colonic epithelial cells that appear in the stool by either intact shedding, necrosis, or apoptosis. Using contemporary DNA or protein degradation technologies, these products, some of which are from neoplastic cells, can be detected and assumed to be of colorectal epithelial origin, unlike gene or protein products detected from blood or urine, which could have arisen from any systemic epithelial source.

Stool testing has many important advantages over other screening strategies. It is noninvasive and samples can be collected in privacy without inconvenience. With mail-in next-day delivery, geographic availability and access are no longer barriers. Patients surveyed about the commercially available versions of stool-based DNA screening tests have found them easy to use and acceptable [13]. Therefore the development of accurate fecal colonocyte DNA and mRNA markers has the potential to revolutionize screening for colorectal cancer.

Cellular Trafficking in the Colonic Crypt

Colonocytes in the crypts have 3-day to 5-day turnover, resulting in the potential shedding into stool of more than 10 billion adult colonic epithelial cells per day, some unknown percentage of which remain viable and trapped in the surface mucous. In the right colon, shed colonocytes in surface mucosa mix with the unformed stool, whereas in the left colon, colonocytes adhere to the surface of the stool, perhaps as the result of physical propulsion along the mucous layer [11]. Many investigators consider normal epithelial renewal to involve apoptosis of normal colono-cytes at the top of the colonic crypts or along the luminal surface between crypts [14]. There is some evidence of phagocytic recycling by macrophages and other evidence of cellular invasion through small colonic pores [15]. The percentage of senescent crypt cells that undergo apoptosis, phagocytic recycling, or cellular involution versus the percentage of crypt cells released into the mucous layer of the lumen remains unknown. Similarly, if viable colonocytes populate the mucous layer, their ability to escape into the lumen of the colon remains unknown (Fig. 1).

In contrast, colorectal neoplasms are more prone to exfoliation because of increased proliferation, less apoptosis, and the decreased cell-cell adhesion inherent in the neoplastic transformation process [16]. The frequent luminal projection and irregularity of polyps and colon cancers increases the surface area and susceptibility to trauma from passing liquid and solid stool. Some argue that in the presence of a neoplasm in the colon, normal colonocytes are outnumbered by neoplastic colonocytes among the shed colonocytes in the stool [12•], but data to support this argument are limited. Despite the higher turnover of colonocytes in a neoplasm, the limited size and surface area of such a lesion compared with the vast expanse of normal mucosa suggests that crypt epithelial turnover most likely contributes the bulk of cells—or at least cell fragments—in stool. Nevertheless, colonic neoplasms shed sufficient DNA into the colon to permit detection of mutations from stool DNA fragments among the sea of DNA from normal colonocytes, food sources, and bacteria [17].

Reliable methods have recently been developed to isolate colonocytes and colorectal cancer cells from spontaneously evacuated stool (not laxative-induced) [18]. New methods for

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human colonocyte mRNA quantification also have been developed, allowing quantification of transcriptional gene expression from these exfoliated colonocytes [19]. These techniques involve the use of reverse transcription real-time polymerase chain reaction (PCR) and can be combined with DNA mutation tests and DNA methylation.

Because exfoliation is likely to be a continuous process, examination of fecal colonocytes will be less prone to sample variation than FOBT and will result in improved sensitivity. It also would eliminate the problem of confounding nonhuman DNA in feces.

Development of Gene-Based Tests in Stool for Detection of Colonic Neoplasms

In 1992, Sidransky et al. [17] reported that *K-ras* mutations were detectable in DNA extracted from exfoliated cells in stool. Because *K-ras* is expressed in less than half of advanced colorectal neoplasia, the sensitivity of this test was low (< 35%), so a single-gene test based on *K-ras* detection is not feasible [17]. Mutations in the adenomatous polyposis coli (*APC*) gene or the *p53* gene are present in up to 70% of colorectal cancers and occur early in the transition from adenoma to cancer, so detecting common mutations in these genes may increase the true-positive rate in combination panels. Mutations were detected in the stool of seven (64%) of 11 patients with known *p53* tumor mutations; among these, three of five FOBT-negative samples had *p53* mutations in the stool [20]. Traverso et al. [21] found *APC* mutations in stool from 61% of patients with early colorectal cancers and 50% with advanced adenomas. No mutations were identified in any of the stool samples from 28 control patients with negative colonoscopies. These data demonstrate that *p53*, *APC*, and *K-ras* mutations from a colonic mutation are detected in DNA fragments from the stool. Unfortunately, true-positive rates are poor, suggesting that it may be necessary to look for different genetic events associated with carcinogenesis to achieve adequate sensitivity and specificity of neoplasm detection (Table 1). Because no single gene mutation has been found to exist in all colonic neoplasia, screening based only on single-gene mutation analysis will yield an unacceptably high false-negative rate. Moreover, the technical efficiency of detecting rare mutations in small DNA fragments from the stool matrix is likely to be less than 100%, further decreasing the diagnostic yield [12•].

Multiplexed-DNA Mutation Panel for Human Stool

The development of more efficient technologies to enable amplification of multiple genes in single-reaction, multiplex PCR technology [22,23] created new opportunities to detect mutations in multiple genes in a single assay (Table 1). Automated methods create the potential for cost-effective tools for mass screening by permitting analytic scale-up of multiple-gene mutation detection [12•]. Several multigene panels have been developed to capitalize on the heterogeneity of alterations in colonic neoplasia for early detection of colorectal cancer [23,24]. These tests required a large volume of stool, from which purified, naked human DNA is obtained using oligonucleotide-based hybrid capture. Realtime PCR is used to detect and quantify the chosen DNA mutation sites. Ahlquist et al. [24] reported a specificity of 93% with sensitivity of 91% for cancer and 82% for adenomas larger than 1 cm while using an assay that included 15 point mutations on *K-ras*, *p53*, and *APC* genes,

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along with Bat26 (a marker of microsatellite instability) and long DNA, in 51 freezer-archived stool specimens [24].

Using a similar multitarget assay panel of 21 specific mutations in *K-ras*, *p53*, and *APC* with an assay for Bat26 and DNA integrity, Tagore et al. [25] found a specificity of 93.6%, a sensitivity of 63.5% for cancer, and a sensitivity of 57.1% for advanced adenomas in 132 patients who provided stool samples prior to colonoscopy. Syngal et al. [26••] showed that these stool DNA mutation abnormalities disappear after excision and treatment of colon cancer, further strengthening a potential role for these panels.

The results of these studies are very promising, but a screening diagnostic test that requires shipment of a bio-sample to a central laboratory poses major problems with logistics, cost, and quality control. To address these issues, a group at Exact Sciences (Marlborough, MA) undertook a population-based screening trial of a multigene fecal DNA diagnostic test ("PreGen-Plus™") compared with FOBT (Hemoccult II). The data of Imperiale et al. [27••] from 4404 patients screened for colorectal cancer report sensitivities for the detection of cancer of 51.6% (95% CI, 34.8%–68%) for the DNA panel and 12.9% (95% CI, 5.1%–28.9%) for Hemoccult II ($P = 0.003$); specificity was 94.4% for the DNA panel and 95.2% for Hemoccult II. When advanced adenoma detection was added to this analysis, the sensitivity of the fecal DNA panel dropped to 40.8% (95% CI, 30.2%–52.5%) while the Hemoccult II sensitivity remained the same at 14.1% (95% CI, 7.8%–24.6%, $P < 0.001$). The fecal DNA panel was not more sensitive than the Hemoccult test in detecting advanced adenomas alone: sensitivity was 15.1% (95% CI, 12.0%–19.0%) for the DNA panel and 10.7% (95% CI, 10.7%–14.1%) for Hemoccult II.

These data support the concept that fecal DNA tests could improve the sensitivity of FOBT and serve as an intermediate, noninvasive screening tool for colorectal adenocarcinoma. Although these tests are more sensitive than single-gene testing, FOBT, and no screening at all, when they were tested in a clinical screening environment of asymptomatic subjects, their sensitivity fell. Moreover, the fecal DNA test did not improve upon FOBT sensitivity for the detection of adenomas, a disappointing outcome. Non-invasive dysplastic cells from large adenomas are known to contain a spectrum of mutations similar to those found in invasive neoplasms [28]. Is the cellular turnover in large adenomas insufficient to generate a mutational signal from DNA in stool? Or are there insufficient DNA mutational events in large adenomas for detection by a limited number of multiplexed mutations available in the stool DNA assay?

Another unexpected outcome in the trial by Imperiale et al. [27••] involved the poor predictive performance of long DNA. Phase II data suggested that long DNA, thought to refect disordered apoptosis, was the most sensitive portion of the PreGen-Plus™ test. Yet in the prospective, multicenter screening trial, long DNA had the poorest predictive performance. These results might be explained by the much longer interval between collection and processing in the large multicenter screening trial compared with the phase II trials. Stool DNA, which can be easily digested by DNAases present in the feces, may have more time to fragment if processing of the sample takes a longer time. Enhanced preservation may reduce DNA degradation, but variable degradation in unformed and

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formed fecal matter prior to defecation may undermine enhanced postdefecation stabilization. The problem of DNA degradation could be eliminated by using intact, viable colonocytes exfoliated into human stool as the source for DNA.

A cost-benefit Markov model by Song et al. [29] assumed the diagnostic sensitivity of the multigene fecal DNA panel to be 65% for adenocarcinoma and 40% for colorectal adenomas, at a base cost estimate of $695 per PreGen-Plus™ test. The model generated a cost of $47,700 per incremental life-year gained, assuming testing every *5* years. For a fecal DNA test to become cost-effective, the base cost would need to be reduced to $195 per test, using sensitivity assumptions 10% higher than the recently published prospective screening trial [30]. On the basis of these data, it is concluded that fecal DNA testing is no more cost-effective than periodic colonoscopy. Given the superior diagnostic performance of colonoscopy, as well as its therapeutic value, routine testing with the currently available multigene fecal DNA panel is not recommended.

DNA Methylation-Based Stool Biomarkers

Epigenetic changes in cellular DNA during carcinogenesis play a major role in cellular transformation. Methylation of tumor suppressor gene promoter sites causes transcriptional silencing, and these genes have potential clinical use as biomarkers and therapeutic targets. At present, cancer-specific DNA methylation changes have focused on CpG islands. These are lengths of DNA about 1 kb long, containing clusters of CpG dinucleotides that are usually located at the 5′ end of a gene and are unmethylated in normal cells. DNA hypomethylation remains largely unexplored in colorectal cancer and could represent another potential area for exploitation.

Aberrantly methylated *CDKN2A* and *MGMT* occur in advanced adenomas, whereas *MLH1* gene methylation remains stable in both tubular adenomas and higher-grade adenomas (Table 1) [31•]. Adenomas in which at least one of these genes was methylated were larger than those with nonmethylated genes (15.6 vs 7 mm; $P = 0.0067$). None of these three genes was methylated in DNA extracted from normal colonic mucosa. In 44 hyperplastic polyps from 17 patients, at least one of these genes was methylated in 20%. Aberrant methylation of these three genes also can be detected in DNA from colon lavage effluent. Unfortunately, the low sensitivity (55%) and specificity (37%) for these methylation assays limit their clinical utility as diagnostic biomarkers for colorectal cancer. This suboptimal performance may be explained by 1) lack of sensitivity of the PCR-based assays in detecting the small amount of polyp DNA in the samples or 2) loss of tumor DNA in the technique used to extract the fecal DNA in the samples that generate false-negative results.

Looking at colon cancers from two independently collected sources, Chen et al. [32••] found that vimen-tin exon-1 expression was methylated in 83% ($n = 46$) and 53% ($n = 107$). This same sequence was found to be unmethylated in 45 of 46 normal colonic tissues tested. In fecal DNA from a separate set of colon cancer patients ($n = 94$), vimentin methylation had a sensitivity of 46% (95% CI, 35%–56%) for detecting colon cancer and 43% (95% CI, 31%–57%) for detecting early-stage tumors. Vimentin methylation of fecal DNA from 198 subjects free of colon cancer was only 10%, giving a specificity of 90% (95% CI, 85%–

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94%). Interestingly, vimentin is not expressed by normal colonic mucosa; the gene encodes a protein constituent of mesenchymal cells. These data suggest that methylation is a widespread event in dysplastic or transformed cells, even in gene promoters that are not specifically known to be important in tumor suppression or cellular differentiation.

The gene for the p110α catalytic subunit of phosphatidylinositol 3-kinase (*PIK3CA*), a key lipid kinase that regulates multiple cellular signaling pathways associated with tumorigenesis, has recently been shown to have a high rate of mutations in colorectal cancer [33•]. The high rate and clustering of mutations in the *PIK3CA* gene would make it a potential marker for early detection.

Recent technological advances have found many ways to identify mutations, including DNA micro-arrays, polymerase chain reaction/ligase detection reaction (PCR/LDR), primer extension assays, hybridization assays, high-performance liquid chromatography (HPLC), single-strand conformational polymorphism analysis, gradient gel electrophoresis, and heteroduplex analysis. Using these technologies to examine shed fecal colonocytes may prove useful in developing a noninvasive screening tool for colorectal cancer.

Conclusions

Fecal DNA testing on shed colonocytes has the potential of providing early detection of colorectal adenomas and cancers that will be accurate, noninvasive, cost-effective, and acceptable to the public. Experience with the Pre-Gen-Plus™ fecal DNA profile of common mutations associated with colorectal carcinogenesis supports the future likelihood that a screening biomarker panel will improve adherence and sensitivity in the early detection of colorectal cancer, but future approaches will require improved sample collection and logistics, as well as reduced cost. The panel of biomarkers needed to optimize sensitivity has yet to be identified.

The targeted population is likely to remain individuals aged 50 and older. The testing interval remains to be determined but probably will be no less than every 5 years, reflecting the current ability of colonoscopy to identify new lesions. The problem of individuals who are positive on screening but negative on colonoscopy will need to be resolved. Will false-positive fecal DNA screening mean that a small island of dysplastic or neoplastic cells is present in the colonic epithelium and was missed by colonoscopy? Or can morphologically normal colonocytes develop mutations with no current or future clinical importance? Can mutated DNA circulating from other organ sites (eg, the biliary tree) be detected in fecal DNA samples? Accurate interpretation of the results of future DNA panels will require clarification of these issues.

References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

• Of importance

•• Of major importance

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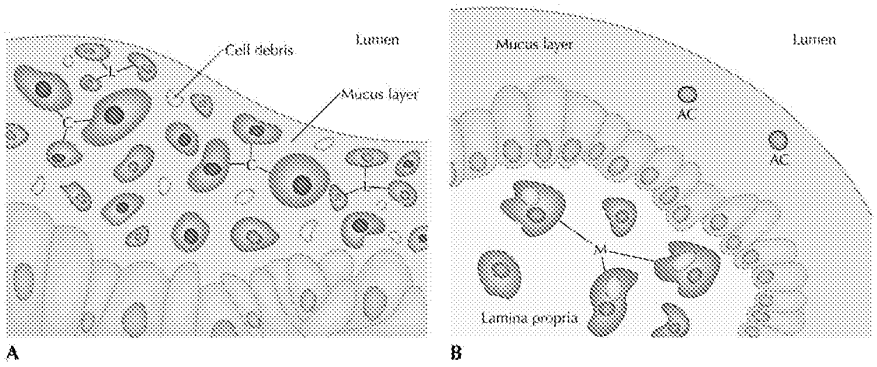


Figure 1.

Possible luminal cellular fluxes at the surface of a colorectal neoplasm (**A**) and normal colonic mucosa (**B**). AC—apoptotic cells; C—viable colonocytes; L—leukocytes; M—macrophages. (*Adapted from* Osborn and Ahlquist [12•], with permission. Copyright © 2005 American Gastroenterological Association.)

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Table 1

Selected dinical validation trials of fecal DNA-based early detection tests for colorectal neoplasia

Study	Marker(s)	Colorectal cancer detection sensitivity, % *(n)*	Adenoma detection sensitivity, % *(n)*	Colorectal cancer detection specificity, % *(n)*	Adenoma detection specificity, % *(n)*
Sidransky et al. [17]	*K-ras*	37 (9/24)	NA	50 (3/6)	NA
Ahlquist et al. [24]	*APC, K-ras, p53;* Bat26; long DNA	91 (20/22)	82 (9/11)	93 (26/28)	82 (9/11)
Tagore et al. [25]	*APC, K-ras, p53;* Bat26; long DNA	63.5 (33/52)	57.1 (16/28)	94.5 (208/220)	98.2 (113/115)
Syngal et al. [26••]	*APC, K-ras, p53;* Bat26; long DNA	54(49/91)	26 (6/23)	NA	NA
Imperiale et al. [27••]	*APC, K-ras,* p53; Bat26; long DNA	52 (16/31)	33 (13/40)	99.3 (2460/2476)	94.4(1344/1423)
Petko et al. [31•]	Aberrantly methylated *CDKN2A*	NA	34(14/41)	NA	100(25/25)
	Aberrantly methylated *MGMT*	NA	49 (19/39)	NA	100 (25/25)
	Aberrantly methylated *MLH1*	NA	7(3/41)	NA	100 (25/25)
Chen et al. [32••]	Methylated vimentin	83 (38/46), 53 (57/107)	NA	90(178/198)	NA
Samuels et al. [33•]	*PIK3CA* mutation clustering	32 (74/119)	2.6 (2/76)	NA	NA

APC—adenomatous polyposis coli; NA—data not available; PIK3CA—phosphatidylinositol 3-kinase p110a catalytic subunit.

Curr Colorectal Cancer Rep. Author manuscript; available in PMC 2015 June 13.

Electronic Patent Application Fee Transmittal

Application Number:	15634607
Filing Date:	27-Jun-2017
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Attorney Docket Number:	EXCTD-35239.304

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:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
		Total in USD ($)		**240**

Electronic Acknowledgement Receipt

EFS ID:	39936127
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:	07-JUL-2020
Filing Date:	27-JUN-2017
Time Stamp:	20:28:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

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1	Information Disclosure Statement (IDS) Form (SB08)	2020-07-07_35239-304_SupplIDS-SB08.pdf	151785 967b30010df51b226ae1ba339867e168057257b2	no	3
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	KIM.pdf	219952 1b535ec52a256030c047834395c29b165ca1cc98	no	8
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Warnings:					
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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.

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APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	REQUEST ID
15/634,607		1634	118041

PAIR Correspondence Address/Fee Address Change

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

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An attorney or Agent of Record registered to practice before the Patent and Trademark Office who has been given power of attorney in this application

Signature:	/Jason R. Bond/
Name:	Jason R. Bond
Registration Number:	45439

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

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An attorney or Agent of Record registered to practice before the Patent and Trademark Office who has been given power of attorney in this application

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Name:	Jason R. Bond
Registration Number:	45439

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

173254 7590 08/10/2020

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
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EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
08/10/2020	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
Genomic_docketing@cardinal-ip.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 05/22/2020 & 07/07/2020.

☐ 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 07/07/2020.

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).