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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/179,945	04/30/2024	11970746	EXCTD-35239.307	2966

173254 7590 04/10/2024

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

ISSUE NOTIFICATION

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

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

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

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

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

Applicant will be able to obtain more detailed information by accessing the Patent Center (https://patentcenter.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

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

Joost Louwagie, Dornach, SWITZERLAND;

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

Exact Sciences Corporation, Madison, WI;

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 USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.



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Document Code:WFEE

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Sale Accounting Date:03/25/2024

Sale Item Reference Number	Effective Date
18179945	03/22/2024

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20243OC11215205	1501	UTILITY APPL ISSUE FEE	$1,200.00	Salea

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**03/22/2024 03:37:40 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Mary Ann Brow
PATENT CENTER #	64801987	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	-

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
web85b.pdf	3	Issue Fee Payment (PTO-85B)	58 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
web85b.pdf	30D58ED640E6E58A283EBF6514DA180267ACCB838CFABD52 9922C8EBE428599020A133EF849AA684A10E833CDC1D00383 F3EDFBF2DE9B00E85F8215C46348B16

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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ISSUE FEE TRANSMITTAL FORM

APPLICATION #	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET #
18179945	03/07/2023	Joost Louwagie	EXCTD-35239.307

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Nonprovisional Application under 35 USC 111(a)	DATE DUE	06/13/2024
CONFIRMATION #	2966	ISSUE FEE DUE	$ 0
EXAMINER	ETHAN WHISENANT	PUBLICATION DUE	$ 0
GROUP ART UNIT	1634	TOTAL FEES DUE	$0
CLASS - SUBCLASS	435/006110	PREV. PAID FEE	$ 1200
ENTITY STATUS	Regular Undiscounted		

1. CHANGE OF CORRESPONDENCE ADDRESS AND/OR INDICATION OF FEE ADDRESS (37 CFR 1.33 & 1.363)

Correspondence Address

CURRENT ADDRESS

Maintenance Fee Address

CURRENT ADDRESS

☐ Change of correspondence address requested,system generated AIA/122-PC form attached

☐ Fee address indication requested, system generatedSB/47-PC form attached

2. ENTITY STATUS

Change in Entity Status

NEW STATUS

3. THE FOLLOWING FEES ARE SUBMITTED

☐ Issue Fee

☑ Publication Fee

4. FEE AUTHORIZATION

☑ I authorize USPTO to apply my previously paid issue fee to the current fees due

☐ The Director is herby authorized to apply my previously paid issue fee to the current fee due and to charge deficient fees to Deposit Account Number:

☑ If in addition to the payment of the issue fee amount submitted with this form, there are any discrepencies in any amount(s) due, the Director is authorized to charge any deficiency, or credit any overpayment, to Deposit Account Number: 504302

5. FIRM AND/OR ATTORNEY NAMES TO BE PRINTED

NOTE: If no name is listed, no name will be printed
For printing on the patent front page, list to be displayed as entered

Casimir Jones, S.C.
Mary Ann D. Brow

6. ASSIGNEE NAME(S) AND RESIDENCE DATA TO BE PRINTED

NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a susbstitute for filing an assignment.

Exact Sciences Corporation	Madison, WI UNITED STATES	Corporation

Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via Patent Center on the date indicated below.

Signature	Name	Registration #	Date
/Mary Ann D. Brow/	Mary Ann Brow	42363	03/22/2024

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	2966
Serial No.:	18/179,945	Group No.:	1634
Filed:	03/07/2023	Examiner:	Ethan C. Whisenant
Entitled:	**FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**		

COMMENTS REGARDING EXAMINER'S STATEMENTS ON AND DOCUMENTS INCLUDED WITH THE CORRECTED NOTICE OF ALLOWABILTY MAILED 03/21/2024

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

Applicant is filing the following comments in response to the Corrected Notice of Allowability mailed 03/21/2024 ("CNOA") for the above-referenced application. Applicant notes with appreciation that the Examiner has corrected the Notice of Allowability mailed 03/13/2024 to correct the numbering of the claims allowed. Applicant agrees with the Examiner's conclusion that claims 1-2 and 4-20 of this application are pending and allowable.

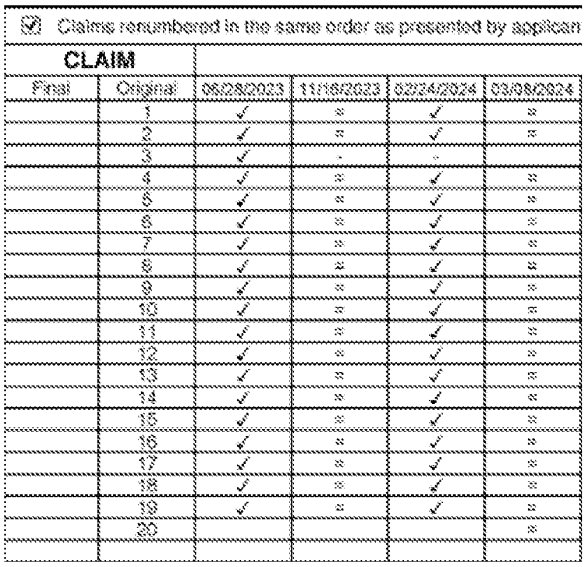
Applicant notes that a list of Attachments on page 2 of the CNOA appears as follows:

Attachment(s)
1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 29 FEB 2024.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

(CNOA, page 2)

Applicant notes that no Information Disclosure Statement was filed in this application on 02/29/2024. However, Applicant notes that a copy of an Information Disclosure Statement filed by the Applicant on 01/16/2024, marked as having been considered by the Examiner on 02/24/2024, and previously mailed to Applicant on 02/29/2024, was attached to the CNOA of 02/21/2024.

Applicant further notes that in the Image File Wrapper, the Index of Claims for this matter dated 03/21/2024 includes the following information pertaining to the claims:

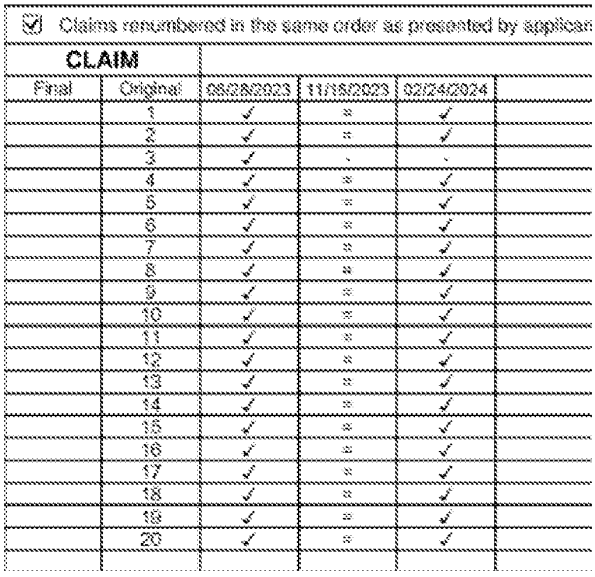


☑ Claims renumbered in the same order as presented by applican

CLAIM					
Final	Original	06/28/2023	11/16/2023	02/24/2024	03/08/2024
	1	✓	=	✓	=
	2	✓	=	✓	=
	3	✓	-	-	
	4	✓	=	✓	=
	5	✓	=	✓	=
	6	✓	=	✓	=
	7	✓	=	✓	=
	8	✓	=	✓	=
	9	✓	=	✓	=
	10	✓	=	✓	=
	11	✓	=	✓	=
	12	✓	=	✓	=
	13	✓	=	✓	=
	14	✓	=	✓	=
	15	✓	=	✓	=
	16	✓	=	✓	=
	17	✓	=	✓	=
	18	✓	=	✓	=
	19	✓	=	✓	=
	20				=

(Index of Claims, 03/21/2024)

Applicant notes that, in the table above, information pertaining to Claim 20 prior to 03/08/2024 is missing. Applicant further notes that, in the Image File Wrapper, an Index of Claims dated 02/29/2024 provides the missing Claim 20 information:



☑ Claims renumbered in the same order as presented by applican

CLAIM					
Final	Original	06/28/2023	11/16/2023	02/24/2024	
	1	✓	=	✓	
	2	✓	=	✓	
	3	✓	-	-	
	4	✓	=	✓	
	5	✓	=	✓	
	6	✓	=	✓	
	7	✓	=	✓	
	8	✓	=	✓	
	9	✓	=	✓	
	10	✓	=	✓	
	11	✓	=	✓	
	12	✓	=	✓	
	13	✓	=	✓	
	14	✓	=	✓	
	15	✓	=	✓	
	16	✓	=	✓	
	17	✓	=	✓	
	18	✓	=	✓	
	19	✓	=	✓	
	20	✓	=	✓	

(Index of Claims, 02/29/2024)

It is Applicant's position that these errors in the CNOA and Image File Wrapper are clear clerical errors that have no bearing on the allowability, scope, interpretation, or any other aspect of any of allowed claims 1-2 and 4-20 of the instant application. Applicant expressly reserves the right to set forth additional and/or alternative reasons for the patentability of the claims in future applications and/or proceedings.

Applicant notes that the Notice of Allowability includes the following statement:

> **Foreign Priority**
>
> ► The applicant has not **CLAIMED** Foreign Priority in this application.

Applicant submits that the priority claimed in the present application is indicated in the Application Data Sheet filed on 03/07/2023, and is reflected on the Filing Receipt mailed 05/22/2023, and that this statement in the CNOA has no bearing on the effective filing date of the application.

It is recognized that, in accordance with M.P.E.P. § 1302.14, the Examiner's reasons for allowance need not set forth all of the details as to why the claims are allowed. In the above-referenced application, it is not conceded that the Examiner's stated reasons for allowance are the only reasons for which the claims are allowable.

Respectfully submitted,

Dated: March 22, 2024 /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

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**03/22/2024 03:34:03 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	64802296	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2024-03-22_35237.307_CommentsExaminersStatement.pdf	3	Post Allowance Communication - Incoming	266 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2024-03-22_35237.307_CommentsExaminersStatement.pdf	6908B2C3E476EA1590BAD3D4AA7D5D10685EB8C110810AAB53E980FBAA8CFF82672ECE21DBE54EAB4960A9219CFD156C93ACD6196218F60556AE104BEE28C496

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

Doc Code: IFEE
Description: Issue Fee Payment (PTO-85B)

PTO/85B



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ISSUE FEE TRANSMITTAL FORM

APPLICATION #	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET #
18179945	03/07/2023	Joost Louwagie	EXCTD-35239.307

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Nonprovisional Application under 35 USC 111(a)	DATE DUE	06/13/2024
CONFIRMATION #	2966	ISSUE FEE DUE	$ 0
EXAMINER	ETHAN WHISENANT	PUBLICATION DUE	$ 0
GROUP ART UNIT	1634	TOTAL FEES DUE	$0
CLASS - SUBCLASS	435/006110	PREV. PAID FEE	$ 1200
ENTITY STATUS	Regular Undiscounted		

1. CHANGE OF CORRESPONDENCE ADDRESS AND/OR INDICATION OF FEE ADDRESS (37 CFR 1.33 & 1.363)

Correspondence Address	Maintenance Fee Address
CURRENT ADDRESS	CURRENT ADDRESS

☐ Change of correspondence address requested,system generated AIA/122-PC form attached

☐ Fee address indication requested, system generatedSB/47-PC form attached

2. ENTITY STATUS

Change in Entity Status

NEW STATUS

3. THE FOLLOWING FEES ARE SUBMITTED

☐ Issue Fee

☑ Publication Fee

4. FEE AUTHORIZATION

☑ I authorize USPTO to apply my previously paid issue fee to the current fees due

☐ The Director is herby authorized to apply my previously paid issue fee to the current fee due and to charge deficient fees to Deposit Account Number:

☑ If in addition to the payment of the issue fee amount submitted with this form, there are any discrepencies in any amount(s) due, the Director is authorized to charge any deficiency, or credit any overpayment, to Deposit Account Number: 504302

5. FIRM AND/OR ATTORNEY NAMES TO BE PRINTED

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For printing on the patent front page, list to be displayed as entered

Casimir Jones, S.C.
Mary Ann D. Brow

6. ASSIGNEE NAME(S) AND RESIDENCE DATA TO BE PRINTED

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Exact Sciences Corporation	Madison, WI UNITED STATES	Corporation

Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via Patent Center on the date indicated below.

Signature	Name	Registration #	Date
/Mary Ann D. Brow/	Mary Ann Brow	42363	03/22/2024

UNITED STATES PATENT AND TRADEMARK OFFICE

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.
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307	2966

173254 7590 03/21/2024

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

EXAMINER	
WHISENANT, ETHAN C	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
03/21/2024	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

Corrected ***Notice of Allowability***	**Application No.** 18/179,945	**Applicant(s)** Louwagie, Joost	
	Examiner Ethan C Whisenant	**Art Unit** 1634	**AIA (FITF) Status** No

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

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

1. ☑ This communication is responsive to 29 FEB 2024.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

3. ☑ The allowed claim(s) is/are 1-2 and 4-20 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

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

   Certified copies:

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

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

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

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

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

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

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 29 FEB 2024.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634

Corrected Notice of Allowability

► **Claims 1-2 and 4-20** as presented in the paper(s) filed 29 FEB 2024 is are pending.

Foreign Priority

►The applicant has not **CLAIMED** Foreign Priority in this application.

REASON(S) FOR ALLOWANCE

► **Claim(s) 1-2 and 4-20** is/are deemed to be allowable in light of the paper(s) filed 29 FEB 2024and the persuasive argument(s) therein. The Claims have now been renumbered as Claims 1-19 in the same order as presented by the applicant.

Conclusion

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

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

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

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

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	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	06/28/2023	11/16/2023	02/24/2024	03/08/2024					
	1	✓	=	✓	=					
	2	✓	=	✓	=					
	3	✓	-	-						
	4	✓	=	✓	=					
	5	✓	=	✓	=					
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	12	✓	=	✓	=					
	13	✓	=	✓	=					
	14	✓	=	✓	=					
	15	✓	=	✓	=					
	16	✓	=	✓	=					
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	18	✓	=	✓	=					
	19	✓	=	✓	=					
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Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

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

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	19	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634		O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20240309

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

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

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

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

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 19	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner) (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

U.S. Patent and Trademark Office Part of Paper No.: 20240309

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	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

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

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	9	10	18	19										
2	2	10	11	19	20										
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3	4	12	13												
4	5	13	14												
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6	7	15	16												
7	8	16	17												
8	9	17	18												

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 19	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner)	(Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

U.S. Patent and Trademark Office Part of Paper No.: 20240309

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12Q 1/68 ; 1/6886 ; 2600/106; 154; 158	06/28/2023	ECW
G01N 33/57446 ; 2333/805 ; 2800/50	06/28/2023	ECW
Updated Search	11/16/2023	ECW
Updated Search	02/24/2024	ECW
Updated Search	03/08/2024	ECW

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

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

Search Notes		
Search Notes	**Date**	**Examiner**
See the Examiner's Search Notes	06/28/2023	ECW
Updated Search	11/16/2023	ECW
Updated Search	02/24/2024	ECW
UpdatedSearch	03/08/2024	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

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	18/179,945	Louwagie, Joost
	Examiner	Art Unit
	Ethan C Whisenant	1634

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
C12Q	1/68 ; 1/6886 ; 2600/106; 154; 158	11/16/2023	ECW
G01N	33/57446 ; 2333/805 ; 2800/50	11/16/2023	ECW
Updated Search		03/08/2024	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	1	of	7	Attorney Docket Number	EXCTD-35239.307

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
		5198365	1993-03-30	GROW et al.	
		5741650	1998-04-21	LAPIDUS et al.	
		5952178	1999-09-14	LAPIDUS et al.	
		7195878	2007-03-27	CLEATOR	
		9891223	2019-02-13	BEAULIEU et al.	
		11634781	2023-04-25	LOUWAGIE	

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
		20060216714	2006-09-28	KANAOKA	
		20120164238	2012-06-28	JOOST	
		20160194723	2016-07-07	LOUWAGIE	

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]
		WO 1997/25925	1997-07-24	HULIOT PLASTICS INDUSTRIES et al.		
		WO 2005/113769	2005-12-01	EXACT SCIENCES CORPORATION		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	02/24/2024

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Sheet	2	of	7	Attorney Docket Number	EXCTD-35239.307

FOREIGN PATENT DOCUMENTS						
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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]
		AHLQUIST et al., A Stool Collection Device: The First Step in Occult Blood Testing, Annals of Internal Medicine, 108(4):609-612 (1988)	
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		Declaration of Brendan T. Jones, dated January 11, 2024, 11 pages	
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				Examiner Name	WHISENANT, Ethan C.
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				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	4	of	7	Attorney Docket Number	EXCTD-35239.307

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				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	5	of	7	Attorney Docket Number	EXCTD-35239.307

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Sheet	6	of	7	Attorney Docket Number	EXCTD-35239.307

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		Recorded Assignment of US Patent No. 11,634,781 dated April 25, 2017, 5 pages	
		USPTO Nonfinal Office Action for US Patent Publication No. US17/936,335 dated January 11, 2023, 7 pages	
		Request for Ex Parte Reexamination of U.S. Patent 11,634,781, filed May 22, 2023, 286 pages	

Examiner Signature		Date Considered	

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				Examiner Name	WHISENANT, Ethan C.
Sheet	7	of	7	*Attorney Docket Number*	EXCTD-35239.307

NONPATENT LITERATURE DOCUMENTS			
		USPTO Order Granting Request for Ex Parte Reexamination of US Patent No. 11,634,781 dated June 29, 2023, 18 pages	
		USPTO Notice of Intent to Issue Ex Parte Reexamination Certificate for US Patent No. 11,634,781 dated Oct 18, 2023, 5 pages	
		Ex Parte Reexamination Certificate for US Patent 11,637,781, issued December 4, 2023, 2 pages	
		Petition for *Inter Parties* Review of U.S. Patent 11,634,781, filed January 11, 2024, 87 pages	

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	02/24/2024

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Sheet	8	of	7	Attorney Docket Number	EXCTD-35239.307

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

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

SIGNATURE

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

Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2024-01-16
Name/Print	Mary Ann D. Brow	Registration Number	42363

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[5] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3).
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This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	2966
Serial No.:	18/179,945	Group No.:	1634
Filed:	03/07/2023	Examiner:	Ethan C. Whisenant
Entitled:	**FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**		

RESPONSE TO NON-FINAL OFFICE ACTION MAILED 02/29/2024

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

This paper is responsive to the Non-Final Office Action mailed 02/29/2024. 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-35239.307**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

The **Status of the Claims** is reflected in the Listing beginning on Page 2; and

Remarks are on Page 6.

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

173254 7590 03/13/2024

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

EXAMINER	
WHISENANT, ETHAN C	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 03/13/2024

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307	2966

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$1200.00	$0	06/13/2024

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

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

HOW TO REPLY TO THIS NOTICE:

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

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

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

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

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

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

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

PTOL-85 (Rev. 11/23)

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

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

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

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

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

173254 7590 03/13/2024
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

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

Certificate of Mailing or Transmission

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

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307	2966

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$1200.00	$0	06/13/2024

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

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

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

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

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

1_____
2_____
3_____

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

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

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

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

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

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

❑ Electronic Payment via the USPTO patent electronic filing system ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

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

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

❑ Applicant certifying micro entity status. See 37 CFR 1.29
NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

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

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

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.
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EXAMINER	
WHISENANT, ETHAN C	
ART UNIT	**PAPER NUMBER**
1634	

DATE MAILED: 03/13/2024

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

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

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

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

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

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

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

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. The United States Patent and Trademark Office (USPTO) collects the information in this record under authority of 35 U.S.C. 2. The USPTO's system of records is used to manage all applicant and owner information including name, citizenship, residence, post office address, and other information with respect to inventors and their legal representatives pertaining to the applicant's/ owner's activities in connection with the invention for which a patent is sought or has been granted. The applicable Privacy Act System of Records Notice for the information collected in this form is COMMERCE/PAT-TM-7 Patent Application Files, available in the Federal Register at 78 FR 19243 (March 29, 2013).
https://www.govinfo.gov/content/pkg/FR-2013-03-29/pdf/2013-07341.pdf

Routine uses of the information in this record may include disclosure to:

1) law enforcement, in the event that the system of records indicates a violation or potential violation of law;
2) a federal, state, local, or international agency, in response to its request;
3) a contractor of the USPTO having need for the information in order to perform a contract;
4) the Department of Justice for determination of whether the Freedom of Information Act (FOIA) requires disclosure of the record;
5) a Member of Congress submitting a request involving an individual to whom the record pertains, when the individual has requested the Member's assistance with respect to the subject matter of the record;
6) a court, magistrate, or administrative tribunal, in the course of presenting evidence, including disclosures to opposing counsel in the course of settlement negotiations;
7) the Administrator, General Services Administration (GSA), or their designee, during an inspection of records conducted by GSA under authority of 44 U.S.C. 2904 and 2906, in accordance with the GSA regulations and any other relevant (i.e., GSA or Commerce) directive, where such disclosure shall not be used to make determinations about individuals;
8) another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c));
9) the Office of Personnel Management (OPM) for personnel research purposes; and
10) the Office of Management and Budget (OMB) for legislative coordination and clearance.

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

Notice of Allowability	Application No. 18/179,945	Applicant(s) Louwagie, Joost	
	Examiner Ethan C Whisenant	Art Unit 1634	AIA (FITF) Status No

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

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

1. ☑ This communication is responsive to the peper(s) filed 29 FEB 2024.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

3. ☑ The allowed claim(s) is/are 1-2 and 4-19 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

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

   Certified copies:

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

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

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

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

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

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

Attachment(s)

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

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634

NOTICE OF ALLOWABILITY

► **Claim(s) 1-2 and 4-19** as presented in the paper(s) filed 29 FEB 2024 is/are pending in this application.

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

REASON(S) FOR ALLOWANCE

► **Claim(s) 1-2 and 4-19** is/are deemed to be allowable in light of the paper(s) filed 29 FEB 2024 06 and the persuasive argument(s) therein. The Claims have now been renumbered as Claims 1-18 in the same order as presented by the applicant.

Conclusion

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

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

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

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

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	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	06/28/2023	11/16/2023	02/24/2024	03/08/2024					
	1	✓	=	✓	=					
	2	✓	=	✓	=					
	3	✓	-	-						
	4	✓	=	✓	=					
	5	✓	=	✓	=					
	6	✓	=	✓	=					
	7	✓	=	✓	=					
	8	✓	=	✓	=					
	9	✓	=	✓	=					
	10	✓	=	✓	=					
	11	✓	=	✓	=					
	12	✓	=	✓	=					
	13	✓	=	✓	=					
	14	✓	=	✓	=					
	15	✓	=	✓	=					
	16	✓	=	✓	=					
	17	✓	=	✓	=					
	18	✓	=	✓	=					
	19	✓	=	✓	=					

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

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

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	18	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	08 March 2024	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20240308

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

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

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

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

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 18	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner)	08 March 2024 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

U.S. Patent and Trademark Office — Part of Paper No.: 20240308

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

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

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	9	10	18	19										
2	2	10	11												
	3	11	12												
3	4	12	13												
4	5	13	14												
5	6	14	15												
6	7	15	16												
7	8	16	17												
8	9	17	18												

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	18	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	08 March 2024	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20240308

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12Q 1/68 ; 1/6886 ; 2600/106; 154; 158	06/28/2023	ECW
G01N 33/57446 ; 2333/805 ; 2800/50	06/28/2023	ECW
Updated Search	11/16/2023	ECW
Updated Search	02/24/2024	ECW
Updated Search	03/08/2024	ECW

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

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

Search Notes		
Search Notes	**Date**	**Examiner**
See the Examiner's Search Notes	06/28/2023	ECW
Updated Search	11/16/2023	ECW
Updated Search	02/24/2024	ECW
UpdatedSearch	03/08/2024	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	Art Unit
	Ethan C Whisenant	1634

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
C12Q	1/68 ; 1/6886 ; 2600/106; 154; 158	11/16/2023	ECW
G01N	33/57446 ; 2333/805 ; 2800/50	11/16/2023	ECW
Updated Search		03/08/2024	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	2966
Serial No.:	18/179,945	Group No.:	1634
Filed:	03/07/2023	Examiner:	Ethan C. Whisenant
Entitled:	**FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**		

RESPONSE TO NON-FINAL OFFICE ACTION MAILED 02/29/2024

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

This paper is responsive to the Non-Final Office Action mailed 02/29/2024. 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-35239.307**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

The **Status of the Claims** is reflected in the Listing beginning on Page 2; and

Remarks are on Page 6.

STATUS OF THE CLAIMS:

1. (original) A method of processing a freshly-collected fecal sample without freezing, the method comprising:

    a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject;

    b) in a sealable vessel, combining a first portion of the fecal sample with a stabilizing buffer, and sealing the sealable vessel; and

    c) in a sealable container, combining a second portion of the fecal sample with a solution that prevents denaturation or degradation of blood proteins found in a fecal sample, and sealing the sealable container.

2. (original) The method of claim 1, further comprising delivering the sealable vessel containing the first portion of the fecal sample and the stabilizing buffer and the sealable container containing the second portion of the fecal sample and the solution to a medical diagnostics laboratory.

3. (canceled)

4. (original) A method of processing a fecal sample, the method comprising:

    a) obtaining a pair of portions of a fecal sample collected from a human subject, the pair of portions comprising:

        i) a sealed sealable vessel containing a first portion of a fecal sample and a stabilizing buffer; and

        ii) a sealed sealable container containing a second portion of a fecal sample and a solution that prevents denaturation or degradation of blood proteins found in a fecal sample,

    wherein the pair of portions are obtained by the method of claim 1;

b) extracting nucleic acid from the first portion of the fecal sample;

c) testing nucleic acid extracted from the first portion of the fecal sample for an amount of a human nucleic acid; and

d) testing the second portion of the fecal sample for an amount of a blood protein present in the second portion of the fecal sample.

5. (original) The method of claim 4, wherein testing the nucleic acid comprises determining expression from a human gene.

6. (original) The method of claim 5, wherein determining expression from the human gene comprises testing the nucleic acid for presence of human DNA having an epigenetic modification.

7. (original) The method of claim 6, wherein testing the nucleic acid for the presence of human DNA having an epigenetic modification comprises measuring an amount of a methylated human DNA.

8. (original) The method of claim 6, wherein the epigenetic modification comprises aberrant methylation.

9. (original) The method of claim 8, wherein the aberrant methylation comprises hypermethylation.

10. (original) The method of claim 6, wherein the human DNA having an epigenetic modification comprises a gene and/or a promoter region of a gene.

11. (original) The method of claim 10, wherein the gene is selected from the group consisting of *PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2,* and *CDO1*.

12. (original) The method of claim 6, wherein testing the nucleic acid for the presence of human DNA having an epigenetic modification comprises modifying the nucleic acid with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.

13. (original) The method of claim 5, wherein determining expression from the human gene comprises measuring an amount of RNA expressed from the human gene.

14. (original) The method of claim 13, wherein measuring an amount of RNA expressed from the human gene comprises reverse transcriptase polymerase chain reaction (RT-PCR).

15. (original) The method of claim 4, wherein testing for an amount of a blood protein present in the second portion comprises testing for a concentration of hemoglobin in the second portion, wherein a concentration of hemoglobin is indicative of a presence of blood in the fecal sample.

16. (original) The method of claim 15, wherein testing for the concentration of hemoglobin comprises immunochemical detection of hemoglobin.

17. (original) The method of claim 15, wherein the second portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the second portion is at least 5 ng/ml.

18. (original) The method of claim 15, wherein the second portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the second portion is at least 10 ng/ml.

19. (original) The method of claim 15, wherein the second portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the second portion is at least 20 ng/ml.

20. (original) The method of claim 15, wherein the second portion of the fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in the second portion is at least 50 ng/ml.

REMARKS

Information Disclosure Statements

Applicant notes with appreciation that the Examiner has considered the references disclosed by the Applicant in the Information Disclosure Statements filed in this matter on 03/07/2023; 06/08/2023; and 01/16/2024.

Claims

Claims 1-2 and 4-20 are pending in the present application, and are listed on pages 2-5 for the Examiner's convenience. No amendments are made in this paper.

Office Action

In an Office Action mailed 02/29/2024, the Examiner rejected all claims on the grounds of non-statutory obviousness-type double patenting over claims 1-14 of U.S. Patent No. 11,845,991 in view of WO 2003/076594 (Sidransky, *et al.*)

Solely in the interest of expediting prosecution, Applicant herewith files a terminal disclaimer over US Patent No. 11,845,991 to obviate the present rejections. Applicant notes that the filing of a terminal disclaimer to obviate a rejection based on non-statutory double patenting is not an admission of the propriety of the rejection.

CONCLUSION

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: February 29, 2024

/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

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**02/29/2024 05:25:39 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	64501184	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 3

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
2024-02-29_ROA_EXCTD-003USCON4-35239-307.pdf		6	-	191 KB
2024-02-29_ROA_EXCTD-003USCON4-35239-307-A....pdf	(1-1)	1	Amendment/Request for Reconsideration-After Non-Final Rejection	133 KB
2024-02-29_ROA_EXCTD-003USCON4-35239-307-CLM.pdf	(2-5)	4	Claims	173 KB
2024-02-29_ROA_EXCTD-003USCON4-35239-307-REM.pdf	(6-6)	1	Applicant Arguments/Remarks Made in an Amendment	168 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2024-02-29_ROA_EXCTD-003USCON4-35239-307.pdf	C294D99952C2459F4F0BED1B111101F3B24818C341ABF7B099066D1871EC97851AE6C0A9029987ACAB2FFB68A387CB1342D52456E0F41BB4A5BB7AA8176BE719
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2024-02-29_ROA_EXCTD-003USCON4-35239-307-CLM.pdf	96DAFE1549A919DBB7C39AD6F438CCBF48B43D4D4C251DB5665BA0358E60A429A4E76D8A92493CFA9F942C9B06F5F552D9EABF3DED0143CAC7BBF562A4FA9921
2024-02-29_ROA_EXCTD-003USCON4-35239-307-REM.pdf	EDC1216DC27FABC116E5980D1D6FAC79CC7F27F61628672DF618006A67655C6477534D12589FD28BF1469344135FB3EF6859EB6E5E6940747CABA7CA3992FC7E

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A PRIOR PATENT

APPLICATION #	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET #
18179945	03/07/2023	Joost Louwagie	EXCTD-35239.307

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

- ☑ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action
- ☑ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

Owner	Percent interest
EXACT SCIENCES CORPORATION, MADISON WI	100%
Total	**100%**

The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s)

Application #	Filing Date

as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.
In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

Patent #
11845991

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.
In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.



Terminal disclaimer fee under 37 CFR 1.20(d) included with Electronic Terminal Disclaimer request.

Applicant claims the following entity status:
Regular Undiscounted

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

I certify, in accordance with 37 CFR 1.4(d)(4) that I am: An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application

Signature	Name	Registration #
/Mary Ann Brow/	Mary Ann Brow	42363

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* Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this certification. See MPEP 324.

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United States Patent and Trademark Office
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APPROVAL LETTER

APPLICATION #	FILING DATE	APPLICANT/PATENT UNDER REEXAMINATION
18/179,945	03/07/2023	Joost Louwagie

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Electronic terminal disclaimer filed on 02/29/2024

☑ Approved

This patent is subject to a Terminal Disclaimer

Approved / Disapproved by: Electronic Terminal Disclaimer automatically approved

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**02/29/2024 04:36:15 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Mary Ann Brow
PATENT CENTER #	64506938	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	-

Documents

TOTAL DOCUMENTS: 2

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
petition-request.pdf	3	Terminal Disclaimer-Filed (Electronic)	44 KB
grantLetter.pdf	1	Terminal Disclaimer-Electronic-Approved	18 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
petition-request.pdf	77146658532314CC3483B4A54DC29E3E87F5A50DE0F6D6028 4078704553BDA0376361A7EB82D95C13C39E5D57924477E734 431F30CD75DCAEF2409C91F6A0BE3
grantLetter.pdf	2D2261750D6A10A7E1B42E5BB189709A15BE56F783C3BD822

DB513DAEED17D30C8BA7F54CAA5184E22489898696CF24BB
5A87FF0069816A2432482AC71F929CB

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New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**02/29/2024 04:36:15 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Mary Ann Brow
PATENT CENTER #	64506938	AUTHORIZED BY	-
CUSTOMER #	173254	FILING DATE	03/07/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Joost Louwagie

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0638	**E20242SG36459150**	**Mary Ann Brow**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1814	STATUTORY DISCLAIMER, INCLUDING TERMINAL DISCLAIMER	170.00	1	170.00
			TOTAL AMOUNT:	**$170.00**

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

New Applications Under 35 U.S.C. 111

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

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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.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307	2966

173254 7590 02/29/2024

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

EXAMINER	
WHISENANT, ETHAN C	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
02/29/2024	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. 18/179,945	Applicant(s) Louwagie, Joost	
	Examiner Ethan C Whisenant	Art Unit 1634	AIA (FITF) Status No

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

Period for Reply

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

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

Status

1)☑ Responsive to communication(s) filed on 07 MAR 2023, 06 OCT2023 and 16 JAN 2024.

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

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

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

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

Disposition of Claims*

5) ☑ Claim(s) 1-2 and 4-20 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-2 and 4-20 is/are rejected.

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

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

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

Application Papers

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

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

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

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

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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

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

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

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

Attachment(s)

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

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 16 JAN 2023.

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

4) ☐ Other: _____.

NON-FINAL OFFICE ACTION

► The applicant's Preliminary Amendment filed 06 OCT 2023 has been entered. Following the entry of the Preliminary Amendment, **Claim(s) 1-2 and 4-20** is/are pending.

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

Non-Statutory Obviousness-type Double Patenting

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

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

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the

form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claim Rejections

► **Claim(s) 1-2, 4-19** is/are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-14 of U.S. Patent No. 11,845,991 [hereinafter "US-991'"] in view of Sirdansky et al. [WO 2003/076594 – hereinafter "Sirdansky"]..

Although the conflicting claims are not identical, they are not patentably distinct from each other. **Claim 1** of the instant application is drawn to a method of processing a freshly-collected fecal sample without freezing, the method comprising: a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject; b) in a sealable vessel, combining a first portion of the fecal sample with a stabilizing buffer, and sealing the sealable vessel; and c) in a sealable container, combining a second portion of the fecal sample with a solution that prevents denaturation or degradation of blood proteins found in a fecal sample, and sealing the sealable container. The Claims of US 991' teach a method of processing a freshly-collected fecal sample without freezing which comprises most of the limitations of Claim 1 except the claims of the instant application require sealing the sealable containers, however absent an unexpected result it would have been *prima facie* obvious to the PHOSITA to seal any sealable containers used to store and/or transport fecal samples to an appropriate diagnostic medical laboratory in order to prevent spillage of said sample.

As regards **Claim 2**, see Claim 2 of US 991.

As regards **Claim 4**, the instant Claim differs from the Claims of US 991' in that the instant claims require that the sealable containers used be sealed. The claims of US 991' teach the claimed method except there is no requirements that the sealable container comprising the fecal sample and stabilizing buffer be sealed, however absent an unexpected result it would have been *prima facie* obvious to the PHOSITA to seal any sealable containers used to store

and/or transport fecal samples to an appropriate diagnostic medical laboratory in order to prevent spillage of said sample.

As regards **Claims 5-14** see Claims 10-14 of US 991'. See also Sirdansky who teach that altered gene expression occurs in CRC which altered expression can be determined by RT-PCR, see at least para 140-145.

As regards **Claims 15-20**, see claims 3-9 of US 991'.

Conclusion

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

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

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

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

Notice of References Cited	Application/Control No. 18/179,945	Applicant(s)/Patent Under Reexamination Louwagie, Joost	
	Examiner Ethan C Whisenant	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date YYYY-MM-DD	Name	CPC Classification	US Classification
*	A	US-11634781-B2	2023-04-25	Louwagie; Joost	G01N33/57446	435/6.1
*	B	US-11845991-B2	2023-12-19	Louwagie; Joost	C12Q1/6886	1/1
	C					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date YYYY-MM-DD	Country	Name	CPC Classification
*	N	WO-03076594-A2	2003-09-18	WIPO	Sirdansky et al.	C12Q1/6827
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

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

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

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	1634

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12Q 1/68 ; 1/6886 ; 2600/106; 154; 158	06/28/2023	ECW
G01N 33/57446 ; 2333/805 ; 2800/50	06/28/2023	ECW
Updated Search	11/16/2023	ECW
Updated Search	02/24/2024	ECW

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

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

Search Notes		
Search Notes	**Date**	**Examiner**
See the Examiner's Search Notes	06/28/2023	ECW
Updated Search	11/16/2023	ECW
Updated Search	02/24/2024	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
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	Ethan C Whisenant	1634

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
C12Q	1/68 ; 1/6886 ; 2600/106; 154; 158	11/16/2023	ECW
G01N	33/57446 ; 2333/805 ; 2800/50	11/16/2023	ECW

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	18/179,945	Louwagie, Joost
	Examiner	**Art Unit**
	Ethan C Whisenant	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	06/28/2023	11/16/2023	02/24/2024						
	1	✓	=	✓						
	2	✓	=	✓						
	3	✓	-	-						
	4	✓	=	✓						
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	18	✓	=	✓						
	19	✓	=	✓						
	20	✓	=	✓						

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
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				First Named Inventor	JOOST LOUWAGIE
				Art Unit	1634
				Examiner Name	WHISENANT, Ethan C.
Sheet	1	of	7	Attorney Docket Number	EXCTD-35239.307

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
		5198365	1993-03-30	GROW et al.	
		5741650	1998-04-21	LAPIDUS et al.	
		5952178	1999-09-14	LAPIDUS et al.	
		7195878	2007-03-27	CLEATOR	
		9891223	2019-02-13	BEAULIEU et al.	
		11634781	2023-04-25	LOUWAGIE	

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
		20060216714	2006-09-28	KANAOKA	
		20120164238	2012-06-28	JOOST	
		20160194723	2016-07-07	LOUWAGIE	

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]
		WO 1997/25925	1997-07-24	HULIOT PLASTICS INDUSTRIES et al.		
		WO 2005/113769	2005-12-01	EXACT SCIENCES CORPORATION		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	02/24/2024

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				Examiner Name	WHISENANT, Ethan C.
Sheet	2	of	7	Attorney Docket Number	EXCTD-35239.307

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		Request for Ex Parte Reexamination of U.S. Patent 11,634,781, filed May 22, 2023, 286 pages	

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				Examiner Name	WHISENANT, Ethan C.
Sheet	7	of	7	*Attorney Docket Number*	EXCTD-35239.307

NONPATENT LITERATURE DOCUMENTS			
		USPTO Order Granting Request for Ex Parte Reexamination of US Patent No. 11,634,781 dated June 29, 2023, 18 pages	
		USPTO Notice of Intent to Issue Ex Parte Reexamination Certificate for US Patent No. 11,634,781 dated Oct 18, 2023, 5 pages	
		Ex Parte Reexamination Certificate for US Patent 11,637,781, issued December 4, 2023, 2 pages	
		Petition for *Inter Parties* Review of U.S. Patent 11,634,781, filed January 11, 2024, 87 pages	

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Sheet	8	of	7	Attorney Docket Number	EXCTD-35239.307

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

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

SIGNATURE

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

Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2024-01-16
Name/Print	Mary Ann D. Brow	Registration Number	42363

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307	2966

173254 7590 01/22/2024

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EXAMINER	
WHISENANT, ETHAN C	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
01/22/2024	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):

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In re Application of	:	
Louwagie, Joost	:	
Application No. 18/179,945	:	
Filed: 7 Mar 2023	:	DECISION ON PETITION
For: FECAL SAMPLE PROCESSING AND	:	
ANALYSIS COMPRISING DETECTION OF	:	
BLOOD	:	

This is a decision on the petition under 37 CFR 1.313(c), filed January 16, 2024, to withdraw the above-identified application from issue after payment of the issue fee.

The petition is **GRANTED**.

The above-identified application is withdrawn from issue for consideration of a submission under 37 CFR 1.114 (request for continued examination). *See* 37 CFR 1.313(c)(2).

Petitioner is advised that the issue fee paid on December 27, 2023, cannot be refunded. If, however, this application is again allowed, petitioner may request that it be applied towards the issue fee required by the new Notice of Allowance.[1]

Telephone inquiries should be directed to the undersigned at (571) 272-3206.

This application is being referred to Technology Center Art Unit 1634 for processing of the request for continued examination under 37 CFR 1.114 and for consideration of the concurrently filed IDS.

/LIANA S WALSH/
Lead Paralegal Specialist, OPET

[1] *The request to apply the issue fee to the new Notice may be satisfied by completing and returning the new Part B – Fee(s) Transmittal Form (along with any balance due at the time of submission). Petitioner is advised that the Issue Fee Transmittal Form* ***must*** *be completed and timely submitted to avoid abandonment of the application.*

PETITION TO WITHDRAW AN APPLICATION FROM ISSUE AFTER PAYMENT OF THE ISSUE FEE UNDER 37 CFR 1.313(c)

First named inventor: LOUWAGIE

Application No.: 18/179,945 Art Unit: 1634

Filed: March 7, 2023 Examiner: WHISENANT

Title: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Attention: Office of Petitions

APPLICANT HEREBY PETITIONS TO WITHDRAW THIS APPLICATION FROM ISSUE UNDER 37 CFR 1.313(c).

A grantable petition requires the following items:

(1)Petition fee; and

(2)One of the following reasons:

(a)Unpatentability of one or more claims, which must be accompanied by an unequivocal statement that one or more claims are unpatentable, an amendment to such claim or claims, and an explanation as to how the amendment causes such claim or claims to be patentable;

(b)Consideration of a request for continued examination in compliance with § 1.114 (for a utility or plant application only); or

(c)Express abandonment of the application. Such express abandonment may be in favor of a continuing application, but not a CPA under 37 CFR 1.53(d).

1. Petition Fee

☒ $140.00(37 CFR 1.17(h)).

2. Reason for withdrawal from issue

☐ One or more claims are unpatentable

☐ Amendment and explanation attached

☒ Consideration of a request for continued examination (RCE)

☒ RCE request attached
☒ RCE fee attached
☒ RCE submission attached

Applicant hereby expressly abandons the instant application (any attorney/agent signing for this reason must have been given power of attorney)

THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR

SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

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

☐An attorney or agent registered to practice before the Patent and Trademark Office

☐A sole inventor

☐A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors

☐A joint inventor; all of whom are signing this e-petition

☐The assignee of record of the entire interest that has properly made itself of record pursuant to 37 CFR 3.71.

Dated: January 16, 2024

/Mary Ann D. Brow/

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	01/16/2024 07:00:05 PM Z ET	EXCTD-35239.307

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	63959858	AUTHORIZED BY	Mary Ann Brow
CUSTOMER #	173254	FILING DATE	03/07/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Joost Louwagie

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0638	E20241FJ02487015	Stephanie Filandrinos

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1801	REQUEST FOR CONTINUED EXAMINATION (RCE) - 1ST REQUEST (SEE 37 CFR 1.114)	1360.00	1	1360.00
1464	PETITIONS REQUIRING THE PETITION FEE SET FORTH IN 37 CFR 1.17(H) (GROUP III)	140.00	1	140.00
			TOTAL AMOUNT:	$1,500.00

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

New Applications Under 35 U.S.C. 111

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

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

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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WO 2009/102788 A2

(54) **Title**: DETECTING NEOPLASM

(57) **Abstract**: This document relates to methods and materials for detecting premalignant and malignant neoplasms. For example, methods and materials for determining whether or not a stool sample from a mammal contains nucleic acid markers or polypeptide markers of a neoplasm are provided.

DETECTING NEOPLASM

BACKGROUND

1. Technical Field

This document relates to methods and materials involved in detecting premalignant and malignant neoplasms (*e.g.*, colorectal and pancreatic cancer).

2. Background Information

About half of all cancer deaths in the United States result from aero-digestive cancer. For example, of the estimated annual cancer deaths, about 25 percent result from lung cancer; about 10 percent result from colorectal cancer; about 6 percent result from pancreas cancer; about 3 percent result from stomach cancer; and about 3 percent result from esophagus cancer. In addition, over 7 percent of the annual cancer deaths result from other aero-digestive cancers such as naso-oro-pharyngeal, bile duct, gall bladder, and small bowel cancers.

SUMMARY

This document relates to methods and materials for detecting premalignant and malignant neoplasms (*e.g.*, colorectal and pancreatic cancer). For example, this document provides methods and materials that can be used to determine whether a sample (*e.g.*, a stool sample) from a mammal contains a marker for a premalignant and malignant neoplasm such as a marker from a colonic or supracolonic aero-digestive neoplasm located in the mammal. The detection of such a marker in a sample from a mammal can allow a clinician to diagnose cancer at an early stage. In addition, the analysis of a sample such as a stool sample can be much less invasive than other types of diagnostic techniques such as endoscopy.

This document is based, in part, on the discovery of particular nucleic acid markers, polypeptide markers, and combinations of markers present in a biological sample (*e.g.*, a stool sample) that can be used to detect a neoplasm located, for example, in a mammal's small intestine, gall bladder, bile duct, pancreas, liver, stomach, esophagus, lung, or naso-oro-pharyngeal airways. For example, as described herein, stool can be analyzed to identify mammals having cancer. Once a particular mammal is determined to have stool containing a neoplasm-specific marker or collection of markers, additional cancer screening techniques can be used to identify the location and nature of the neoplasm. For example, a stool sample

can be analyzed to determine that the patient has a neoplasm, while magnetic resonance imaging (MRI), endoscopic analysis, and tissue biopsy techniques can be used to identify the location and nature of the neoplasm. In some cases, a combination of markers can be used to identify the location and nature of the neoplasm without additional cancer screening techniques such as MRI, endoscopic analysis, and tissue biopsy techniques.

In general, one aspect of this document features a method of detecting pancreatic cancer in a mammal. The method comprises, or consists essentially of determining the ratio of an elastase 3A polypeptide to a pancreatic alpha-amylase polypeptide present within a stool sample. The presence of a ratio greater than about 0.5 indicates that the mammal has pancreatic cancer. The presence of a ratio less than about 0.5 indicates that the mammal does not have pancreatic cancer.

In another aspect, this document features a method of detecting pancreatic cancer in a mammal. The method comprises or consists essentially of determining the level of an elastase 3A polypeptide in a stool sample from the mammal. The presence of an increased level of an elastase 3A polypeptide, when compared to a normal control level, is indicative of pancreatic cancer in the mammal.

In another aspect, this document features a method of detecting pancreatic cancer in a mammal. The method comprises, or consists essentially of, determining the level of a carboxypeptidase B polypeptide in a stool sample from the mammal. An increase in the level of a carboxypeptidase B polypeptide, when compared to a normal control level, is indicative of pancreatic cancer in the mammal.

In another aspect, this document features a method of detecting pancreatic cancer in a mammal. The method comprises, or consists essentially of, determining whether or not a stool sample from the mammal comprises a ratio of a carboxypeptidase B polypeptide to a carboxypeptidase A2 polypeptide that is greater than about 0.5. The presence of the ratio greater than about 0.5 indicates that the mammal has pancreatic cancer.

In another aspect, this document features a method of detecting cancer or pre-cancer in a mammal. The method comprises, or consists essentially of, determining whether or not a stool sample from the mammal has an increase in the number of DNA fragments less than 200 base pairs in length, as compared to a normal control. The presence of the increase in the number of DNA fragments less than 200 base pairs in length indicates that the mammal has cancer or pre-cancer. The DNA fragments can be less than 70 base pairs in length.

In another aspect, this document features a method of detecting colorectal cancer or pre-cancer in a mammal. The method comprises, or consists essentially of, determining

Geneoscopy Exhibit 1067, Page 3

Exact Sciences Ex. 2005, Page 93 of 760

whether or not a stool sample from the mammal has an elevated K-ras (Kirsten rat sarcoma-2 viral (v-Ki-ras2) oncogene homolog (GenBank accession no. NM_033360; gi|34485724|)) mutation score, an elevated BMP3 (bone morphogenetic protein 3 (GenBank accession no. M22491; gi|179505)) methylation status, and an elevated level of human DNA as compared to a normal control. The presence of the elevated K-ras mutation score, elevated BMP3 methylation status, and elevated level of human DNA level indicates that the mammal has colorectal cancer or pre-cancer. The K-ras mutation score can be measured by digital melt curve analysis. The K-ras mutation score can be measured by quantitative allele specific PCR.

In another aspect, this document features a method of detecting aero-digestive cancer or pre-cancer in a mammal. The method comprises, or consists essentially of, determining whether or not a stool sample from the mammal has an elevated K-ras mutation score, an elevated BMP3 methylation status, and an elevated level of human DNA as compared to a normal control. The presence of the elevated K-ras mutation score, elevated BMP3 methylation status, and elevated level of human DNA level indicates that the mammal has aero-digestive cancer or pre-cancer. The K-ras mutation score can be measured by digital melt curve analysis. The K-ras mutation score can be measured by quantitative allele-specific PCR. The method can further comprise determining whether or not a stool sample from the mammal has an elevated APC mutation score. The APC mutation score can be measured by digital melt curve analysis.

In another aspect, this document features a method of detecting aero-digestive cancer or pre-cancer in a mammal. The method comprises, or consists essentially of, determining whether or not the mammal has at least one mutation in six nucleic acids selected from the group consisting of p16, p53, k-ras, APC (adenomatosis polyposis coli tumor suppressor (GenBank accession no. NM_000038; gi|189011564)), SMAD4 (SMAD family member 4 (GenBank accession no. NM_005359; gi|195963400)), EGFR (epidermal growth factor receptor (GenBank accession no. NM_005228; gi|41327737|)), CTNNB1 (catenin (cadherin-associated protein), beta 1 (88kD) (GenBank accession no. X87838; gi|1154853|)), and BRAF (B-Raf proto-oncogene serine/threonine-protein kinase (p94) (GenBank accession no. NM_004333; gi|187608632|)) nucleic acids. The presence of at least one mutation in each of the six nucleic acids indicates that the mammal has aero-digestive cancer or pre-cancer. The method can further comprise determining whether or not a stool sample from the mammal has an elevated level of a carboxypeptidase B polypeptide as compared to a normal control. The presence of the elevated level of a caboxypeptidase B polypeptide indicates that the

Geneoscopy Exhibit 1067, Page 4

Exact Sciences Ex. 2005, Page 94 of 760

mammal has aero-digestive cancer or pre-cancer in the mammal. The method can further comprise determining whether or not a stool sample from the mammal has an elevated amount of DNA fragments less than 70 base pairs in length as compared to a normal control. The presence of the elevated amount of DNA fragments less than 70 base pairs in length indicates that the mammal has aero-digestive cancer or pre-cancer. The method can further comprise determining whether or not a stool sample from the mammal has an elevated amount of DNA fragments greater than 100 base pairs in length as compared to normal controls. The presence of the elevated amount of DNA fragments greater than 100 base pairs in length indicates that the mammal has aero-digestive cancer or pre-cancer. The method can further comprise determining whether or not a stool sample from the mammal has an elevated BMP3 methylation status. The elevated BMP3 methylation status level indicates that the mammal has aero-digestive cancer or pre-cancer. The determining step can comprise using digital melt curve analysis.

In another aspect, this document features a method of detecting aero-digestive cancer or pre-cancer in a mammal. The method comprises, or consists essentially of, measuring mutations in a matrix marker panel in a stool sample. The marker panel can comprise measuring DNA mutations in p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF nucleic acids. The presence of a mutation in each of the nucleic acids is indicative of the presence of aero-digestive cancer or pre-cancer in a mammal.

In another aspect, this document features a method of detecting aero-digestive cancer in a mammal. The method comprises, or consists essentially of, determining whether or not the methylation status of an ALX4 (aristaless-like homeobox 4 (GenBank accession no. AF294629; gi|10863748|)) nucleic acid in a stool sample from the mammal is elevated, as compared to a normal control. The presence of an elevated ALX4 methylation status indicates the presence of aero-digestive cancer in the mammal.

In another aspect, this document features a method of diagnosing pancreatic cancer in a mammal. The method comprises, or consists essentially of, obtaining a stool sample from the mammal, determining the ratio of an elastase 3A polypeptide to a pancreatic alpha-amylase polypeptide present within a stool sample, and communicating a diagnosis of pancreatic cancer if the ratio is greater than about 0.5, thereby diagnosing the mammal with pancreatic cancer.

In another aspect, this document features a method of diagnosing a mammal with pancreatic cancer. The method comprises, or consists essentially of, obtaining a stool sample from the mammal, measuring mutations in a matrix marker panel of nucleic acids present in

Geneoscopy Exhibit 1067, Page 5

Exact Sciences Ex. 2005, Page 95 of 760

the sample, determining the ratio of a carboxypeptidase B polypeptide to a carboxypeptidase A2 polypeptide present within the sample, and communicating a diagnosis of pancreatic cancer or pre-cancer if a mutation is detected in each of the marker panel nucleic acids and the ratio is greater than 0.5, thereby diagnosing the mammal. The matrix marker panel comprises or consists essentially of p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF nucleic acids.

In another aspect, this document features method of diagnosing a mammal with colorectal cancer. The method comprises, or consists essentially of, obtaining a stool sample from the mammal, detecting mutations in a matrix marker panel comprising of p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF nucleic acids in DNA present within the sample, measuring the level of a serotransferrin polypeptide present within the sample, and communicating a diagnosis of colorectal cancer or pre-cancer if a mutation is detected in each of the nucleic acids and the level of a serotransferrin polypeptide is elevated as compared to a reference level, thereby diagnosing the mammal.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1: Adjusted Cut-off Levels with Quantitative Stool Markers to Achieve 95% Specificity across Age and Gender using the Q-LEAD Model. Solid line for women, dotted line for men.

Figure 2: Sensitive and specific detection of pancreatic cancer by fecal ratio of carboxypeptidase B:carboxypeptidase A2. Note that ratio in stools from patients with colorectal cancer is no different from ratios with healthy controls.

Figure 3: Elastase levels quantified in stools from patients with pancreatic cancer, patients with colorectal cancer, and healthy controls.

Figure 4: Ratio of elastase3A:pancreatic alpha amylase differentiates patients with pancreatic cancer from patients with colorectal cancer and from healthy controls.

Figure 5: Digital Melt Curve to detect mutations by targeted gene scanning (temperature (x-axis) v. temperature-normalized fluorescence (y-axis)). Eight pairs of primers, which amplify 100-350 bp gene fragments, were used to scan K-ras and APC genes and detect mutations (substitution and deletion mutations, respectively) at 1% mutant/wild-type ratio.

Figure 6: Quantitative detection of low abundance mutations by Digital Melt Curve Assay. Varying Mutant:Wild-type ratios of K-ras and APC gene mixtures were prepared and assayed blindly by Digital Melt Curve.

Figure 7: High analytical sensitivity by Digital Melt Curve (temperature (x-axis) v. temperature-normalized fluorescence (y-axis)). To test the detection limit of digital melt curve (DMC) assay, mutant copies were spiked in wild-type copies at 0.1, 0.5, 1, 5, and 10% dilutions. DMC could detect up to 0.1% mutant/wild-type level when 1000 copies were dispersed to one 96-well plate. The numbers of positive wells increased proportionally when spiked mutant copies were increased. A pair of primers that amplify 248 bp K-ras gene fragment were used as an example here. Primers that amplify 119 bp K-ras gene, 162 bp APC gene, and 346 bp APC genes were also used to test the detection limit and quantitative property of DMC.

Figure 8: Superior screen detection of colorectal precancerous polyps by Digital Melt Curve (DMC). Histogram compares sensitivity by DMC with that by common fecal occult blood tests (Hemoccult and HemoccultSENSA) and by the commercial stool DNA test (PreGenPlus, Exact Sciences). Detection by DMC was significantly better than by any other test ($p<0.05$).

Figure 9: Distributions of short fragment human DNA (short DNA) and long fragment human DNA (long DNA) in stools from patients with normal colonoscopy, large precancerous adenomas, and colorectal cancer. Human DNA quantified by an assay of Alu repeats. Short DNA represents 45 bp fragment amplification products, and long DNA represents 245 bp amplification products.

Figure 10: Stool distributions of short and long DNA in patients with pancreatic cancer and in healthy controls. Short DNA represents 45 bp fragment amplification products, and long DNA represents 245 bp amplification products.

Geneoscopy Exhibit 1067, Page 7

Exact Sciences Ex. 2005, Page 97 of 760

Figure 11: Methylated BMP3 gene in stool for detection of colorectal neoplasia. Methylated BMP3 was blindly quantified in stools from patients with colorectal cancers, precancerous adenomas, and normal individuals with real-time methylation-specific PCR. Each circle represents a stool sample.

Figure 12: Frequency of Specific Base Changes in Colorectal Tumors.

DETAILED DESCRIPTION

This document provides methods and materials related to detecting a neoplasm in a mammal (*e.g.*, a human). For example, this document provides methods and materials for using nucleic acid markers, polypeptide markers, and combinations of markers present in a biological sample (*e.g.*, a stool sample) to detect a neoplasm in a mammal. Such a neoplasm can be a cancer or precancer in the head and neck, lungs and airways, esophagus, stomach, pancreas, bile ducts, small bowel, or colorectum. It will be appreciated that the methods and materials provided herein can be used to detect neoplasm markers in a mammal having a combination of different neoplasms. For example, the methods and materials provided herein can be used to detect nucleic acid and polypeptide markers in a human having lung and stomach neoplasms.

In some cases, the methods and materials provided herein can be used to quantify multiple markers in biological samples (*e.g.*, stool) to yield high sensitivity for detection of lesions (*e.g.*, neoplasms), while preserving high specificity. Such methods can include, for example, a logistic model that adjusts specificity cut-offs based on age, gender, or other variables in a target population to be tested or screened.

In some cases, the methods and materials provided herein can be used to determine whether a mammal (*e.g.*, a human) has colorectal cancer or pancreatic cancer. For example, serotransferin, methylated BMP3, and mutant BRAF markers in stool can be used to identify a mammal as likely having colorectal cancer, while mutant p16, carboxypeptidase B/A, and elastase 2A markers can be used to identify a mammal as likely having pancreatic cancer.

Any suitable method can be used to detect a nucleic acid marker in a mammalian stool sample. For example, such methods can involve isolating DNA from a stool sample, separating out one or more particular DNAs from the total DNA, subjecting the DNAs to bisulfite treatment, and determining whether the separated DNAs are abnormally methylated (*e.g.*, hypermethylated or hypomethylated). In some cases, such methods can involve isolating DNA from a stool sample and determining the presence or absence of DNA having a particular size (*e.g.*, short DNA). It is noted that a single stool sample can be analyzed for

Geneoscopy Exhibit 1067, Page 8

Exact Sciences Ex. 2005, Page 98 of 760

one nucleic acid marker or for multiple nucleic acid markers. For example, a stool sample can be analyzed using assays that detect a panel of different nucleic acid markers. In addition, multiple stool samples can be collected from a single mammal and analyzed as described herein.

Nucleic acid can be isolated from a stool sample using, for example, a kit such as the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA). In addition, nucleic acid can be isolated from a stool sample using the following procedure: (1) homogenizing samples in an excess volume (>1:7 w:v) of a stool stability buffer (0.5M Tris pH 9.0, 150mM EDTA, 10mM NaCl) by shaking or mechanical mixing; (2) centrifuging a 10 gram stool equivalent of each sample to remove all particulate matter; (3) adding 1 μL of 100 μg/μL RNase A to the supernatant and incubating at 37°C for 1 hour; (4) precipitating total nucleic acid with 1/10 volume 3M NaAc and an equal volume isopropanol; and (5) centrifuging and then resuspending the DNA pellet in TE (0.01 M Tris pH 7.4, 0.001 M EDTA). U.S. Patents 5,670,325; 5,741,650; 5,928,870; 5,952,178, and 6,020,137 also describe various methods that can be used to prepare and analyze stool samples.

One or more specific nucleic acid fragments can be purified from a nucleic acid preparation using, for example, a modified sequence-specific hybrid capture technique (see, *e.g.*, Ahlquist *et al.* (2000) *Gastroenterology*, 119:1219-1227). Such a protocol can involve: (1) adding 300 μL of sample preparation to an equal volume of a 6 M guanidine isothiocyanate solution containing 20 pmol biotinylated oligonucleotides (obtained from, for example, Midland Certified Reagent Co., Midland, TX) with sequences specific for the DNA fragments to be analyzed; (2) incubating for two hours at 25°C; (3) adding streptavidin coated magnetic beads to the solution and incubating for an additional hour at room temperature; (4) washing the bead/hybrid capture complexes four times with 1X B+W buffer (1 M NaCl, 0.01 M Tris-HCl pH 7.2, 0.001 M EDTA, 0.1% Tween 20); and (5) eluting the sequence specific captured DNA into 35 μL L-TE (1 mM Tris pH 7.4, 0.1 M EDTA) by heat denaturation of the bead/hybrid capture complexes. Any other suitable technique also can be used to isolate specific nucleic acid fragments.

Nucleic acid can be subjected to bisulfite treatment to convert unmethylated cytosine residues to uracil residues, while leaving any 5-methylcytosine residues unchanged. A bisulfite reaction can be performed using, for example, standard techniques: (1) denaturing approximately 1 μg of genomic DNA (the amount of DNA can be less when using micro-dissected DNA specimens) for 15 minutes at 45°C with 2 N NaOH; (2) incubating with 0.1 M hydroquinone and 3.6 M sodium bisulfite (pH 5.0) at 55°C for 4-12 hours; (3) purifying

Geneoscopy Exhibit 1067, Page 9

Exact Sciences Ex. 2005, Page 99 of 760

the DNA from the reaction mixture using standard (*e.g.*, commercially-available) DNA miniprep columns or other standard techniques for DNA purification; (4) resuspending the purified DNA sample in 55 μL water and adding 5 μl 3 N NaOH for a desulfonation reaction that typically is performed at 40°C for 5-10 minutes; (5) precipitating the DNA sample with ethanol, washing the DNA, and resuspending the DNA in an appropriate volume of water. Bisulfite conversion of cytosine residues to uracil also can be achieved using other methods (*e.g.*, the CpGenome™ DNA Modification Kit from Serologicals Corp., Norcross, GA).

Any appropriate method can be used to determine whether a particular DNA is hypermethylated or hypomethylated. Standard PCR techniques, for example, can be used to determine which residues are methylated, since unmethylated cytosines converted to uracil are replaced by thymidine residues during PCR. PCR reactions can contain, for example, 10 μL of captured DNA that either has or has not been treated with sodium bisulfite, 1X PCR buffer, 0.2 mM dNTPs, 0.5 μM sequence specific primers (*e.g.*, primers flanking a CpG island within the captured DNA), and 5 units DNA polymerase (*e.g.*, Amplitaq DNA polymerase from PE Applied Biosystems, Norwalk, CT) in a total volume of 50 μl. A typical PCR protocol can include, for example, an initial denaturation step at 94°C for 5 min, 40 amplification cycles consisting of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, and a final extension step at 72°C for 5 minutes.

To analyze which residues within a captured DNA are methylated, the sequences of PCR products corresponding to samples treated with and without sodium bisulfite can be compared. The sequence from the untreated DNA will reveal the positions of all cytosine residues within the PCR product. Cytosines that were methylated will be converted to thymidine residues in the sequence of the bisulfite-treated DNA, while residues that were not methylated will be unaffected by bisulfite treatment.

Purified nucleic acid fragments from a stool sample or samples can be analyzed to determine the presence or absence of one or more somatic mutations. Mutations can be single base changes, short insertion/deletions, or combinations thereof. Methods of analysis can include conventional Sanger based sequencing, pyrosequencing, next generation sequencing, single molecule sequencing, and sequencing by synthesis. In some cases, mutational status can be determined by digital PCR followed by high resolution melting curve analysis. In other cases, allele specific primers or probes in conjunction with amplification methods can be used to detect specific mutations in stool DNA. The mutational signature can comprise not only the event of a base or sequence change in a specific gene, but also the location of the change within the gene, whether it is coding, non-coding,

Geneoscopy Exhibit 1067, Page 10

Exact Sciences Ex. 2005, Page 100 of 760

synonymous or non-synonymous, a transversion or transition, and the dinucleotide sequence upstream and downstream from the alteration.

In some cases, a sample can be assessed for the presence or absence of a polypeptide marker. For example, any appropriate method can be used to assess a stool sample for a polypeptide marker indicative of a neoplasm. For example, a stool sample can be used in assays designed to detect one or more polypeptide markers. Appropriate methods such as those described elsewhere (Aebersold and Mann, *Nature*, 422:198-207 (2003) and McDonald and Yates, *Dis. Markers*, 18:99-105 (2002)) can be adapted or designed to detect polypeptides in a stool. For example, single-reaction monitoring using a TSQ mass spectrometer can specifically target polypeptides in a stool sample. High resolution instruments like the LTQ-FT or LTQ orbitrap can be used to detect polypeptides present in a stool sample.

The term "increased level" as used herein with respect to the level of an elastase 3A polypeptide is any level that is above a median elastase 3A polypeptide level in a stool sample from a random population of mammals (*e.g.*, a random population of 10, 20, 30, 40, 50, 100, or 500 mammals) that do not have an aero-digestive cancer. Elevated polypeptide levels of an elastase 3A polypeptide can be any level provided that the level is greater than a corresponding reference level. For example, an elevated level of an elastase 3A polypeptide can be 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more fold greater than the reference level of elastase 3A polypeptide in a normal sample. It is noted that a reference level can be any amount. For example, a reference level for an elastase 3A polypeptide can be zero. In some cases, an increased level of an elastase 3A polypeptide can be any detectable level of an elastase 3A polypeptide in a stool sample.

The term "increased level" as used herein with respect to the level of an carboxypeptidase B polypeptide level is any level that is above a median carboxypeptidase B polypeptide level in a stool sample from a random population of mammals (*e.g.*, a random population of 10, 20, 30, 40, 50, 100, or 500 mammals) that do not have an aero-digestive cancer. Elevated polypeptide levels of carboxypeptidase B polypeptide can be any level provided that the level is greater than a corresponding reference level. For example, an elevated level of carboxypeptidase B polypeptide can be 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more fold greater than the reference level carboxypeptidase B polypeptide observed in a normal stool sample. It is noted that a reference level can be any amount. For example, a reference level for a carboxypeptidase B polypeptide can be zero. In some cases, an

Geneoscopy Exhibit 1067, Page 11

Exact Sciences Ex. 2005, Page 101 of 760

increased level of a carboxypeptidase B polypeptide can be any detectable level of a carboxypeptidase B polypeptide in a stool sample.

The term "increased level" as used herein with respect to the level of DNA fragments less than about 200 or less than about 70 base pairs in length is any level that is above a median level of DNA fragments less than about 200 or less than about 70 base pairs in length in a stool sample from a random population of mammals (*e.g.*, a random population of 10, 20, 30, 40, 50, 100, or 500 mammals) that do not have an aero-digestive cancer. In some cases, an increased level of DNA fragments less than about 200 or less than about 70 base pairs in length can be any detectable level of DNA fragments less than about 200 or less than about 70 base pairs in length in a stool sample.

The term "elevated methylation" as used herein with respect to the methylation status of a BMP3 or ALX nucleic acid is any methylation level that is above a median methylation level in a stool sample from a random population of mammals (*e.g.*, a random population of 10, 20, 30, 40, 50, 100, or 500 mammals) that do not have an aero-digestive cancer. Elevated levels of BMP3 or ALX methylation can be any level provided that the level is greater than a corresponding reference level. For example, an elevated level of BMP3 or ALX methylation can be 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more fold greater than the reference level methylation observed in a normal stool sample. It is noted that a reference level can be any amount.

The term "elevated mutation score" as used herein with respect to detected mutations in a matrix panel of particular nucleic acid markers is any mutation score that is above a median mutation score in a stool sample from a random population of mammals (*e.g.*, a random population of 10, 20, 30, 40, 50, 100, or 500 mammals) that do not have an aero-digestive cancer. An elevated mutation score in a matrix panel of particular nucleic acid markers can be any score provided that the score is greater than a corresponding reference score. For example, an elevated score of K-ras or APC mutations can be 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more fold greater than the reference score of K-ras or APC mutations observed in a normal stool sample. It is noted that a reference score can be any amount.

In some cases, a ratio of particular polypeptide markers can be determined and used to identify a mammal having an aero-digestive cancer (*e.g.*, a colorectal cancer or a pancreatic cancer). For example, a ratio provided herein (*e.g.,* the ratio of carboxypeptidase B polypeptide levels to carboxypeptidase A2 polypeptide levels) to can be used as described herein to identify a mammal having a particular neoplasm (*e.g.,* pancreatic cancer).

Geneoscopy Exhibit 1067, Page 12

Exact Sciences Ex. 2005, Page 102 of 760

In some cases, a matrix marker panel can be used to identify mammals having an aero-digestive cancer (*e.g.*, a colorectal cancer or a pancreatic cancer). In some cases, such panel also can identify the location of the aero-digestive cancer. Such a panel can include nucleic acid markers, polypeptide markers, and combinations thereof and can provide information about a mutated marker gene, the mutated region of the marker gene, and/or type of mutation. For example, data can be analyzed using a statistical model to predict tumor site (*e.g.*, anatomical location or tissue of origin) based on inputs from sequencing data (such as by specific nucleic acid or combination of nucleic acids mutated, specific mutational location on a nucleic acid, and nature of mutation (*e.g.* insertion, deletion, transition, or transversion) or by any combination thereof) and/or data from polypeptide or other types of markers. For example, a Site of Tumor Estimate (SITE) model can be used to predict tumor site using a matrix panel of markers that are present to variable extent across tumors.

In some cases, data can be analyzed using quantified markers to create a logistic model, which can have both high sensitivity and high specificity. For example, a logistic model can also incorporate population variables like gender and age to adjust cut-off levels for test positivity and thereby optimize assay performance in a screening setting. In some cases, a Quantitative Logistic to Enhance Accurate Detection (Q-LEAD) Model can be used with any marker class or combination of markers as long as they can be quantified.

This document also provides methods and materials to assist medical or research professionals in determining whether or not a mammal has an aero-digestive cancer. Medical professionals can be, for example, doctors, nurses, medical laboratory technologists, and pharmacists. Research professionals can be, for example, principle investigators, research technicians, postdoctoral trainees, and graduate students. A professional can be assisted by (1) determining the ratio of particular polypeptide markers in a stool sample, and (2) communicating information about the ratio to that professional, for example. In some cases, a professional can be assisted by (1) determining the level of human DNA, the methylation status of genes such as BMP3, and the mutation score of genes such as APC and K-ras, and (2) communicating information about the level of DNA, the methylation status of particular genes, and the mutation score of particular genes to the professional. In some cases, a professional can be assisted by (1) detecting mutations in cancer-related genes such as K-ras, p53, APC, p16, EGFR, CTNNB1, BRAF, and SMAD4, as a matrix marker panel, and (2) communicating information regarding the mutations to the professional.

After the ratio of particular polypeptide markers, or presence of particular nucleic acid markers in a stool sample is reported, a medical professional can take one or more actions

Geneoscopy Exhibit 1067, Page 13

Exact Sciences Ex. 2005, Page 103 of 760

that can affect patient care. For example, a medical professional can record the results in a patient's medical record. In some cases, a medical professional can record a diagnosis of an aero-digestive cancer, or otherwise transform the patient's medical record, to reflect the patient's medical condition. In some cases, a medical professional can review and evaluate a patient's entire medical record, and assess multiple treatment strategies, for clinical intervention of a patient's condition. In some cases, a medical professional can record a tumor site prediction with the reported mutations. In some cases, a medical professional can request a determination of the ratio of particular polypeptide markers to predict tumor site. In some cases, a medical professional can review and evaluate a patient's entire medical record and assess multiple treatment strategies, for clinical intervention of a patient's condition.

A medical professional can initiate or modify treatment of an aero-digestive cancer after receiving information regarding a ratio of particular polypeptide markers or the presence of nucleic acid markers in a patient's stool sample. In some cases, a medical professional can compare previous reports and the recently communicated ratio of particular polypeptide markers, or presence of nucleic acid markers, and recommend a change in therapy. In some cases, a medical professional can enroll a patient in a clinical trial for novel therapeutic intervention of an aero-digestive cancer. In some cases, a medical professional can elect waiting to begin therapy until the patient's symptoms require clinical intervention.

A medical professional can communicate the ratio of particular polypeptide markers to a patient or a patient's family. In some cases, a medical professional can provide a patient and/or a patient's family with information regarding aero-digestive cancers, including treatment options, prognosis, and referrals to specialists, *e.g.*, oncologists and/or radiologists. In some cases, a medical professional can provide a copy of a patient's medical records to communicate the ratio of particular polypeptide markers to a specialist.

A research professional can apply information regarding a subject's ratio of particular polypeptide markers to advance aero-digestive cancer research. For example, a researcher can compile data on the ratio of particular polypeptide markers, and/or presence of particular nucleic acid markers, with information regarding the efficacy of a drug for treatment of aero-digestive cancer to identify an effective treatment. In some cases, a research professional can obtain a subject's ratio of particular polypeptide markers, and/or determine the presence of particular nucleic acid markers to evaluate a subject's enrollment, or continued participation in a research study or clinical trial. In some cases, a research professional can classify the severity of a subject's condition, based on the ratio of particular polypeptide markers and/or the levels of particular nucleic acid markers. In some cases, a research professional can

Geneoscopy Exhibit 1067, Page 14

Exact Sciences Ex. 2005, Page 104 of 760

communicate a subject's ratio of particular polypeptide markers, and/or the presence of particular nucleic acid markers to a medical professional. In some cases, a research professional can refer a subject to a medical professional for clinical assessment of an aero-digestive cancer, and treatment of an aero-digestive cancer.

Any appropriate method can be used to communicate information to another person (*e.g.*, a professional). For example, information can be given directly or indirectly to a professional. For example, a laboratory technician can input the ratio of particular polypeptide markers and/or particular nucleic acid markers into a computer-based record. In some cases, information is communicated by making a physical alteration to medical or research records. For example, a medical professional can make a permanent notation or flag a medical record for communicating a diagnosis to other medical professionals reviewing the record. In addition, any type of communication can be used to communicate the information. For example, mail, e-mail, telephone, and face-to-face interactions can be used. The information also can be communicated to a professional by making that information electronically available to the professional. For example, the information can be communicated to a professional by placing the information on a computer database such that the professional can access the information. In addition, the information can be communicated to a hospital, clinic, or research facility serving as an agent for the professional.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Multi-Marker Quantitation and a Q-LEAD Model

Most approaches at marker detection in stool have been qualitative. When such qualitative approaches are applied to assay of multiple markers (targeting multiple markers is required with neoplasm detection due molecular heterogeneity), sensitivity is achieved at the expense of compounded non-specificity. Non-specificity can lead to prohibitive programmatic cost with population screening due to the expensive and unnecessary evaluations of false-positive tests. However, if markers are quantified, then a logistic model can be created to achieve both high sensitivity and high specificity. Such a logistic model can also incorporate population variables like gender and age to adjust cut-off levels for test positivity and thereby optimize assay performance in a screening setting (Figure 1). This

Geneoscopy Exhibit 1067, Page 15

Exact Sciences Ex. 2005, Page 105 of 760

Quantitative Logistic to Enhance Accurate Detection (Q-LEAD) Model can be used with any marker class or combination of markers as long as they can be quantified.

A combination of more than one marker was undertaken to achieve the desired sensitivity and specificity for cancer detection. Binary regression methods predicting disease as a function of diagnostic tests estimate the optimal combination of the tests for classifying a subject as diseased or not. McIntosh and Pepe, *Biometrics* 58: 657-664 (2002). A logistic regression model can assess the relationship between a binary dependent response variable such as presence or absence of disease and one or more independent predictor variables. The independent predictors may be qualitative (*e.g.*, binary) or quantitative (*e.g.*, a continuous endpoint). In the Q-LEAD model, the independent predictors can include such biological markers as K-ras, BMP3 and DNA concentration, and others. Importantly, the model incorporates the demographic variables of gender and age, as we have observed that both age and gender influence molecular marker levels in stool. As average stool marker levels increase with age and male gender, failure to adjust for these variables would yield suboptimal specificity in men and elderly persons tested. Coefficients are estimated from the sample data for each term in the model. The result of the model is a risk score for each subject. Cutoffs for predicting disease state from this risk score can be determined in order to maximize sensitivity and specificity of the marker combinations for predicting disease as desired. The inclusion of demographic variables allows these cutoffs to be determined as a function of age and gender.

As an application of the Q-LEAD Model, the following was performed to evaluate a quantitative stool DNA assay approach targeting three informative markers for the detection of colorectal neoplasia. Subjects included 34 with colorectal cancer, 20 with adenomas >1 cm, and 26 with normal colonoscopy. Subjects added a DNA stabilization buffer with stool collection, and stools were frozen at -80°C within 72 hours. From thawed stool aliquots, crude DNA was extracted by standard methods, and target genes were enriched by sequence capture. K-ras mutation score, methylation of BMP3 gene, and concentration of human DNA (245 bp length) were respectively quantified by a digital melt curve assay, real-time methylation-specific PCR, and real-time Alu PCR, respectively. Assays were performed blinded. A logistic model, which incorporates three markers and gender, was constructed to analyze discrimination by combined markers.

Age medians were 60 for patients with colorectal cancer, 66 for those with adenomas, and 61 for normal controls; and male/female distributions were 23/11, 9/11, and 10/16, respectively. Detection rates of colorectal neoplasms were determined by individual

quantitative markers at specificity cut-offs of 96 percent and by combined markers (Table 1). Discrimination by combined markers was calculated using a qualitative binomial method (each marker considered as positive or negative based on individual 96 percent specificity) and by the Q-LEAD model (sensitivity data shown at overall specificity of 96 percent).

Table 1. Specificity and sensitivity of cancer markers.

Specificity		Sensitivity		
		Cancers	Adenomas	Both
Individual Markers				
k-ras mutation	96%	42%	32%	38%
BMP3 methylation	96%	45%	32%	40%
DNA concentration	96%	65%	40%	56%
Combined Markers				
Qualitative method	88%	90%	58%	78%
Q-LEAD Model	96%	90%	47%	76%

By quantitative assay and multivariable analysis of an informative marker panel, stool DNA testing can achieve high sensitivity while preserving high specificity for detection of colorectal neoplasia. The particular three-marker combination of mutant K-ras, BMP3 methylation, and human DNA concentration represents a complementary, high-yield panel.

The above data set and additional data were analyzed as follows. A quantitative stool DNA assay approach targeting four informative markers for use in the detection of colorectal cancer and advanced adenoma was evaluated. Subjects comprised 74 patients with colorectal cancer, 27 with an adenoma >1 cm, and 100 with normal colonoscopy. Stools were collected with a stabilization buffer before or >1 week after colonoscopy and were frozen at -80°C within 24 hours of collection. From thawed stool aliquots, crude DNA was extracted as described above, and target genes were enriched by sequence-specific capture. Human DNA concentration, K-ras and APC mutation scores, and BMP3 methylation were sensitively quantified by real-time Alu PCR, a digital melt curve assay (Zou *et al.*, *Gastroenterology*, "High Detection Rates of Colorectal Neoplasia by Stool DNA Testing With a Novel Digital Melt Curve Assay," (2008)), and real-time methylation-specific PCR, respectively. Assays were performed blindly. Sensitivities and specificities of single makers and their combinations were analyzed.

Geneoscopy Exhibit 1067, Page 17

Exact Sciences Ex. 2005, Page 107 of 760

Age medians were 61 for patients with colorectal cancer, 67 for those with adenomas, and 59 for normal controls; and, male/female ratios were 52/22, 15/12, and 37/63, respectively. The table displays detection rates of colorectal neoplasms by individual quantitative markers at specificities of 90% and by combined markers at two specificities. (Table 2) Data in this table represent a training set and have not been adjusted for age and gender. Yet, it is clear that the full panel of Alu, K-ras, APC, and BMP3 detected more neoplasms than any individual marker, $p<0.05$. At 90% specificity, the full panel detects more adenomas >3 cm (90%, 9/10) than ≤3 cm (47%, 8/17), $p<0.05$, and more colorectal cancers at stages III-IV (89%, 40/45) than at stages I-II (69%, 20/29) $p<0.05$. Neoplasm detection rates were not affected by tumor location.

Table 2. Specificity and sensitivity of a four marker panel

Specificity		**Sensitivity**			
		Colorectal cancers		**Adenomas**	
		I-II	III-IV	≤ 3 cm	> 3 cm
Individual Markers					
APC mutation	90%	38%	40%	47%	50%
k-ras mutation	90%	46%	42%	24%	50%
BMP3 methylation	90%	36%	38%	12%	30%
DNA concentration	90%	52%	76%	41%	50%
Combined Markers					
	90%	69%	89%	47%	90%,

In conclusion, a quantitative stool DNA assay system that incorporates a stabilization buffer with specimen collection, high analytical sensitivity, and a panel of broadly informative markers can achieve high detection rates of both colorectal cancers and advanced adenoma.

Example 2 - SITE model and matrix marker panel

A statistical model (Site of Tumor Estimate (SITE)) can be used to predict tumor site (*e.g.*, anatomical location or tissue of origin) based on inputs from sequencing data (such as by specific nucleic acid or combination of nucleic acids mutated, specific mutational location on a nucleic acid, and nature of mutation (*e.g.* insertion, deletion, transition, or transversion) or by any combination thereof) and/or data from polypeptide or other types of markers.

Geneoscopy Exhibit 1067, Page 18

Exact Sciences Ex. 2005, Page 108 of 760

A matrix marker panel was developed to include eight cancer-related genes: K-ras, p53, APC, p16, EGFR, CTNNB1, BRAF, and SMAD4. The mutation frequencies of these genes were tabulated against the six major aero-digestive cancers based on literature or public database reviews and on actual sequencing observations (Table 3). Literature frequencies were derived from the COSMIC somatic mutation database, review articles, and texts.

Table 3. Matrix Panel of Markers by Tumor Site

AD Cancer Site		p16	p53	K-ras	APC	SMAD4	EGFR	CTNNB1	BRAF	
Literature										
Colorectal		<5%	50-75%	40%	85%	14%	NA	13%	20%	
Pancreatic		85-100%	50-60%	80-90%	10-40%	30%	NA	3-8%	<5%	
Lung		15-25%	25-75%	20-40%	5%	7%	30%	6%	<5%	
Bile Duct		15-60%	30-60%	40%	30-40%	17%	NA	1%	14%	
Gastric		5-30%	20-50%	10%	20-60%	NA	<1%	30-50%	<5%	
Esophageal		5-90%	40-90%	5-12%	5-60%	NA	NA	1%	<5%	
Actual (non-dbSNP)	N									Total Unique
Colorectal	57	5%	47%	26%	75%	25%	12%	2%	30%	98%
Pancreatic	24	29%	17%	62%	54%	8%	8%	4%	8%	83%
Lung	56	9%	57%	9%	16%	14%	14%	2%	4%	77%
Bile Duct	15	13%	27%	13%	20%	13%	0	0	7%	67%
Gastric	23	17%	22%	4%	35%	17%	4%	4%	0	65%
Esophageal	24	4%	46%	4%	33%	17%	4%	0	4%	79%

Some of the frequencies include other genetic alterations than simply single base changes and small insertions/deletions such as methylation events, large homozygous deletions, and copy number changes. Such alterations would not be reflected in the actual frequency table. Actual frequencies were derived by sequencing coding and flanking gene regions from 245 patient tissue samples reflecting the spectrum of aero-digestive cancers. Only non-synonymous and splice site alterations were tabulated. When specific mutational hot-spot sites were able to be identified for particular genes, only those sites were analyzed.

The matrix panel includes markers that are present to variable extent across these tumors so that their aggregate use achieves high overall sensitivity and allows prediction of tumor site using the SITE Model. 70% of tumors harbored one or more mutations from the eight gene panel. Some gene mutations, like those associated with p16, are common in

Geneoscopy Exhibit 1067, Page 19

Exact Sciences Ex. 2005, Page 109 of 760

tumors above the colon but rare for those in the colon. Mutant K-ras is frequent with colorectal and pancreatic cancers but infrequent in the other cancers. Mutations in EGFR clustered with lung and colorectal tumors and mutations in SMAD4 clustered with stomach and colorectal tumors. Genes such as p53, are commonly mutated across many different types of cancers, but specific mutational locations or types of mutations within p53 and other genes differ between tumor site (*e.g.*, Greenman *et al.*, *Nature*, 446(7132):153-8 (2007); Soussi and Lozano, *Biochem. Biophys. Res. Commun.*, 331(3):834-42 (2005); Stephens *et al.*, *Nat. Genet.*, 37(6):590-2 (2005); Sjoblom *et al.*, *Science*, 314(5797):268-74 (2006); Wood *et al.*, *Science*, 318(5853):1108-13 (2007); and Davies, *Cancer Res.*, 65(17):7591-5 (2005)) and can be factored in to the SITE Model to predict tumor site. Single base substitutions were the most common type of mutation throughout the panel and those that predicted colorectal tumors included C-G and A-T transversions (Figure 12). Other tumor sites had similarly unique base change profiles. (Table 4). Insertion/deletions mutations were most common with colorectal tumors, particularly adenomas.

Table 4: Specific Base Change Fractions in AD Tumors

TUMOR	C>T (G>A)	T>C (A>G)	G>C (C>G)	A>T (T>A)	G>T (C>A)	T>G (A>C)
Head and Neck	0.38	0.12		0.38		0.12
Esophageal	0.8	0.07			0.13	
Lung	0.3	0.11	0.02	0.13	0.34	0.09
Stomach	0.5	0.25		0.17	0.08	
Pancreas	0.41	0.15	0.04	0.07	0.33	
Bile Duct	0.5		0.12	0.25	0.12	
CRA	0.34	0.05	0.11	0.11	0.34	0.03
CRC	0.4	0.05	0.02	0.24	0.26	0.03

Polypeptide markers found in stool, such as by proteomic approaches, can also be used to detect aero-digestive neoplasms and predict tumor site. The following was performed to identify and explore candidate polypeptide markers in stool for the discriminate detection of pancreatic cancer. Subjects included 16 cases with pancreatic cancer, 10 disease controls (colorectal cancer), and 24 healthy controls. Whole stools were collected and frozen promptly in aliquots at -80°C. Thawed aliquots were centrifuged, and the aqueous supernatant from each was analyzed. Polypeptides were separated by 1-D electrophoresis, excised from gels, and digested for mass spectrometric analysis using an LTQ-Orbitrap. Data outputs were searched using Mascot, Sequest, and X!Tandem programs against an updated

Geneoscopy Exhibit 1067, Page 20

Exact Sciences Ex. 2005, Page 110 of 760

Swissprot database that included all cataloged species. Unique peptide counts and ratio calculations were performed using Scaffold software.

Median age for pancreatic cancer cases was 67, for colorectal cancer controls 63, and for healthy controls 62; and male/female distributions were 9/7, 6/4, and 9/15, respectively. Using shotgun-proteomic techniques on stools, two pancreatic enzymes (carboxypeptidases B and A2) were conspicuous, as unique spectral counts of the former were commonly elevated with pancreatic cancer and of the latter commonly decreased. Considered together as the ratio of carboxypeptidase B/carboxypeptidase A2, pancreatic cancer cases were almost completely separated from colorectal cancer and healthy control groups. Median ratios were 0.9, 0.2, and 0.3, respectively. At a specificity cut-off for the carboxypeptidase B/A2 ratio at 100% (i.e., ratios from normal control and colorectal cancer stools all below cut-off), sensitivity for pancreatic cancer was 86 percent (Figure 2). Only two pancreatic cancers were misclassified.

These results demonstrate that a stool assay of polypeptide markers can be a feasible non-invasive approach to the detection pancreatic cancer. These results also demonstrate that multivariable analysis of specific polypeptide ratios can be used.

In addition, polypeptide markers unique to colorectal neoplasms were identified (Table 5). For example, serotransferrin was found in stools from patients with colorectal cancer but not in those with pancreatic cancer. These markers when considered as part of a matrix panel contribute both to overall sensitivity for tumor detection and help discriminate colorectal from pancreatic cancer.

Table 5. Positive Stool Findings.

	Carboxypeptidase B/A2*	Serotransferrin
Colorectal Cancer	0	60%
Pancreatic Cancer	86%	0
Normal controls	0	0

*ratio > 0.75 considered positive

Another polypeptide in stool that is pancreatic cancer specific is elastase 3A. Methods and Results demonstrating this are as follows:

Stool Preparation

Samples were collected in phosphate buffered saline and either dropped off in clinic or mailed in collection tub. Samples were homogenized and frozen within 72 hours after receipt. Frozen stools were diluted 1:3 w:v in PBS (Roche, Cat# 1666789). Diluted stools were stomached in a filter bag (Brinkman, BA6041/STR 177 x 305 mm) for 60 seconds on control setting and spun at 10,000 rpms for 30 minutes. Following an additional 10 minute spin at 14,000 rpm, the supernatant was filtered through a 0.45-μm syringe filter and analyzed. Total protein present in stool was quantitated using a Bradford Protein Assay kit (Pierce).

1-Dimensional Electrophoresis

Stool supernatants were diluted 1:1 in Leammli-BME buffer and run on a 10.5-14 % gradient gel. Vertical slices were cut from 250 kDa to 15 kDa and in-gel digested using methods described elsewhere (*e.g.*, Wilm *et al*., *Nature*, 379:466-469 (1996)). Bands were destained, dehydrated, digested in trypsin, extracted, and lyophilized for MS analysis.

Mass Spectrometry

Lyophilized samples were reconstituted and injected with a flow of 500 nL/min and a 75 minute gradient from 5-90% 98% acetonitrile. MS was performed in data dependent mode to switch automatically between MS and MS^2 acquisition on the three most abundant ions. Survey scans were acquired with resolution r = 60,000 at 40 m/z using FWHM with a target accumulation of 10^6 counts. An isolation width of 2.5 m/z was applied. Exclusion mass width was 0.6 m/z on low end and 1.5 m/x on high end. All acquisition and method development was performed using Xcaliber version 2.0.

Database Searching all ms/ms

Samples were analyzed using Mascot (Matrix Science, London, UK; version 2.1.03), Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12) and X! Tandem (World Wide Web at "thegpm.org"; version 2006.09.15.3). Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM. Sequest was searched with a fragment ion mass tolerance of 1.00 Da. Nitration of tyrosine was specified in Mascot as a variable modification.

Criteria for Polypeptide Identification

Scaffold (version Scaffold-01_06_06, Proteome Software Inc., Portland, OR) was used to validate MS/MS based polypeptide identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller *et al.*, *Anal. Chem.*, 74(20):5383-92 (2002)). Polypeptide identifications were accepted if they could be established at greater than 99.0 percent probability and contained at least two identified peptides. Polypeptide probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, *Anal. Chem.*, 75(17):4646-58 (2003)). Polypeptides that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Specific to Elastase 3A ratio determination

Ratios of elastase 3A were determined using spectral counts for each polypeptide. Ratios were determined by dividing the number of unique peptides of elastase 3A (determined using a composite ID from database search modules Mascot, XTandem, and Seaquest and compiled in Scaffold) by the number of unique peptides from another polypeptide such as pancreatic alpha-amylase.

Results

The concentration of a specific pancreatic enzyme, elastase 3A, was consistently found to be elevated in the fecal supernatant of patients with pancreatic cancer as compared to normal controls or patients with non-pancreatic cancer (Figure 3). These finding indicate that fecal concentration of elastase 3A is an accurate marker for pancreatic cancer. In addition, the ratio of elastase 3A against other pancreatic enzymes (or other stable fecal polypeptides) was found to be especially discriminant for pancreatic cancer and obviates the need to determine absolute elastase 3A concentrations (Figure 4). While mass spectrometry was used to make these observations, elastase 3A levels and ratios including elastase 3A can be measured using other methods as well.

Example 3 - Digital Melt Curve Assay for Scanning Mutations

A sensitive, rapid, and affordable method for scanning mutations in bodily fluids at high-throughput was developed. A melt curve assay is a post-PCR technique that can be used to scan for mutations in PCR amplicons. Mutations in PCR products can be detected by changes in the shape of the melting curve (heterozygote from mutant sample) compared to a reference sample (homozygote from wild-type sample) (Figure 5). Melt curve assay can scan

Geneoscopy Exhibit 1067, Page 23

Exact Sciences Ex. 2005, Page 113 of 760

all mutations in a DNA fragment <400 bp in less than 10 minutes, rather than individually targeting single mutations. Regular melt curve assays can detect mutations down to a limit of 5% mutant:wild-type and, thus, are not sensitive enough to detect mutations in many biological samples. For instance, in stool, an analytical sensitivity of 1% or less is required in order to detect precancerous polyps or small early stage cancers. Importantly, a quantitative score can be given to density of target mutations (Figure 6).

Digital PCR can augment the sensitivity of PCR to detect low abundance mutations. Gene copies can be diluted and distributed into 96 wells of a plate to increase the percentage of mutant copy to wild-type copies in certain wells. For example, if a stool DNA sample contains only 1% of mutant BRAF copies compared to wild-type copies, distributing 300 copies of BRAF gene into a 96-well plate can lead to three wells with an average mutant ratio of 33 percent (1:3). After PCR amplification, these three wells with mutant copies can be detected by sequencing or other approaches. Since digital PCR requires PCR on a whole 96-well plate and 96 sequencings (or other approaches) for each target, it can be slow and costly.

The concept of digital melt curve assay is to combine the scanning ability and speed of high resolution melt curve assay with the sensitivity of digital PCR. Miniaturizing and automating this technology dramatically lowers per assay cost and achieves high-throughput necessary for population screening.

The following procedure was used to perform a digital melt curve assay. To prepare a DNA sample, gene target fragments (*e.g.*, BRAF, K-ras, APC, p16, etc.) were captured from stool DNA using a sequence-specific capture method and were quantified with real-time PCR. About 200 to 2000 gene copies were mixed in tube with all PCR reagents. An average of 2 to 20 copies (variable) were distributed to each well of a 96-well plate. PCR amplification was performed using specific primers on the plate (*e.g.*, one target per plate). Final concentrations of PCR mastermix for Digital Melt Curve assays in a 96-well plate (500 μL dispersed to 96 wells with each well containing 5 μL) were as follows: 2X pfx amplification buffer (Invitrogen), 0.3 mM each dNTP, 200 nM forward primer, 200 nM reverse primer, 1 mM $MgSO_4$, 0.02 unit/μL Platinum® pfx polymerase (Invitrogen), and 0.1 unit/μL LcGreen+ dye (Idaho Tech). A high resolution melt curve assay was used to identify the wells with mutant copies. Sequencing was optionally performed to confirm 1 to 2 representative wells.

In some cases, emulsion PCR can be used in place of digital PCR. In such cases, each lipid drop can become a tiny PCR reactor of one single molecule of gene.

Geneoscopy Exhibit 1067, Page 24

Exact Sciences Ex. 2005, Page 114 of 760

Table 6: Sequence Specific Capture Probes and DNA Primers

Gene	Target Region	Capture probe/ primer	Oligo Sequence (5'→3')	SEQ ID No.
KRAS	Codons 12/13	Probe	GTGGACGAATATGATCCAACAATAGAGGTAAATCTTG	1
	Codons 12/13	Primer 1	AGGCCTGCTGAAAATGACTG	2
			TTGTTGGATCATATTCGTCCAC	3
	Codons 12/13	Primer 2	TAAGGCCTGCTGAAAATGAC	4
			ATCAAAGAATGGTCCTGCAC	5
	Codons 12/13	Primer 3	CGTCTGCAGTCAACTGGAATTT	6
			TGTATCGTCAAGGCACTCTTGC	7
	Codons 12/13	Primer 4	CTTAAGCGTCGATGGAGGAG	8
			TTGTTGGATCATATTCGTCCAC	3
BRAF	V600E	Probe	CCAGACAACTGTTCAAACTGATGGGACCCACTCCATC	9
	V600E	Primer	CCACAAAATGGATCCAGACA	10
			TGCTTGCTCTGATAGGAAAATG	11
APC	MCR	Probe 1	CAGATAGCCCTGGACAAACCATGCCACCAAGCAGAAG	12
	MCR	Probe 2	TTCCAGCAGTGTCACAGCACCCTAGAACCAAATCCAG	13
	MCR	Probe 3	ATGACAATGGGAATGAAACAGAATCAGAGCAGCCTAAAG	14
	Codons 1286-1346	Primer 1	TTCATTATCATCTTTGTCATCAGC	15
			CGCTCCTGAAGAAAATTCAA	16
	Codons 1346-1367	Primer 2	TGCAGGGTTCTAGTTTATCTTCA	17
			CTGGCAATCGAACGACTCTC	18
	Codons 1394-1480	Primer 3	CAGGAGACCCCACTCATGTT	19
			TGGCAAAATGTAATAAAGTATCAGC	20
	Codons 1450-1489	Primer 4	CATGCCACCAAGCAGAAGTA	21
			CACTCAGGCTGGATGAACAA	22
	Codon 1554	Primer 5	GAGCCTCGATGAGCCATTTA	23
			TCAATATCATCATCATCTGAATCATC	24
	102457delC	Primer 6	GTGAACCATGCAGTGGAATG	25
			ACTTCTCGCTTGGTTTGAGC	26
	102457delC	Primer 7	CAGGAGACCCCACTCATGTT	19
			CATGGTTTGTCCAGGGCTAT	27
	102457delC	Primer 8	GTGAACCATGCAGTGGAATG	25
			AGCATCTGGAAGAACCTGGA	28
TP53	Exon 4	Probe	AAGACCCAGGTCCAGATGAAGCTCCCAGAATGCCAGA	29
	Exon 4	Primer	CCCTTCCCAGAAAACCTACC	30
			GCCAGGCATTGAAGTCTCAT	31
	Exon 5	Probe	CATGGCCATCTACAAGCAGTCACAGCACATGACGGAG	32
	Exon 5	Primer	CACTTGTGCCCTGACTTTCA	33
			AACCAGCCCTGTCGTCTCT	34
	Exon 6	Probe	AGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGAC	35
	Exon 6	Primer	CAGGCCTCTGATTCCTCACT	36
			CTTAACCCCTCCTCCCAGAG	37
	Exon 7	Probe	ATGTGTAACAGTTCCTGCATGGGCGGCATGAACCGGA	38
	Exon 7	Primer	CTTGGGCCTGTGTTATCTCC	39
			GGGTCAGAGGCAAGCAGA	40
	Exon 8	Probe	CGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGC	41
	Exon 8	Primer	GGGAGTAGATGGAGCCTGGT	42

			GCTTCTTGTCCTGCTTGCTT	43

Example 4 – Sensitive Detection of Mutations using a Digital Melt Curve Assay

The following was performed to develop a quantitative method for scanning gene mutations and to evaluate the sensitivity of the quantitative method for detecting target mutations in stool. A digital melt curve assay was designed by combining digital PCR to a modified melt curve assay. Target genes in low concentration were PCR amplified with a saturated DNA dye, LcGreen+, in a 96-well plate. Each well contained a small number of gene copies, which allowed high mutation/wild-type ratios in some wells that were then detected by melt curve scanning using a LightScanner. Mutations were scored based on the number of wells containing mutant copies in a 96-well plate. To test sensitivity, mutant genes were spiked into a wild-type pool at 0.1, 0.5, 1, 5, and 10% dilutions, and analyzed using digital melt curve assay with 250-1000 gene copies per 96-well plate. This method was then applied in the stool detection of APC, p53, K-ras, and BRAF mutations from 48 patients known to have mutations in one of these genes in matched tumor tissue. Subjects included 9 patients with pancreatic cancer, 31 with colorectal cancer, and 8 with colorectal adenoma >1 cm. All mutations detected by digital melt curve were further confirmed by Sanger sequencing.

The digital melt curve assay detected as few as 0.1% mutant copies for amplicons <350 bp using one 96-well plate (Figure 7), compared to the detection limit for regular melt curve of ≥5 percent. Each mutation scanning took 8-10 minutes with this manual approach. Mutations of APC, p53, K-ras, and BRAF genes were all successfully scanned with digital melt curve in quantitative fashion. Tissue-confirmed mutations were detected from matched stools in 88 percent (42/48) of patients with gastrointestinal neoplasms, including 89 percent with pancreatic cancer, 90 percent with colorectal cancer, and 75 percent with colorectal adenoma >1 cm.

These results demonstrate that a digital melt curve assay can be a highly sensitive approach for detecting mutations in stool, and that it has potential for diagnostic application with both upper and lower gastrointestinal neoplasms.

Example 5 – Using a Digital Melt Curve Assay to Detect Adenomas

Archived stools were used to evaluate a digital melt curve assay of DNA markers for detection of advanced adenomas and to compare the accuracy of the digital melt curve assay with that of occult blood testing and a commercial DNA marker assay method (EXACT

Sciences). Average risk subjects collected stools without a preservative buffer and mailed them to central processing laboratories for banking and blinded stool testing by Hemoccult, HemoccultSENSA, and DNA marker assay. All subjects underwent a colonoscopy, and tissue from advanced adenomas was archived. Archival stools were selected from the 27 patients with a colorectal adenoma >1 cm found to harbor mutant K-ras on tissue analyses and from the first 25 age and gender matched subjects with normal colonoscopy. Standard methods were used to extract crude DNA from fecal aliquots, and K-ras gene was enriched by sequence capture. Mutations in the K-ras gene were quantified by a digital melt curve assay based on the number of wells containing mutant gene copies in a 96-well plate and confirmed by sequencing.

Median age with adenomas was 67 and controls 71; and males/females were 12/15 and 13/14, respectively. Median adenoma size was 1.5 cm (range 1-3 cm). Based on a cut-off of >3 wells with mutant K-ras, the digital melt curve assay yielded an overall sensitivity of 59 percent for adenomas with a specificity of 92 percent; sensitivity for adenomas >2 cm was 80 percent (8/10) and for those <2 cm was 47 percent (8/17), p=0.1. In these same stools, overall adenoma detection rates were 7 percent by Hemoccult, 15 percent by HemoccultSENSA, and 26 percent by the EXACT Sciences K-ras assay ($p<0.05$ for each vs. digital melt curve) (Figure 8). Respective specificities were 92 percent, 92 percent, and 100 percent.

These results demonstrate that an analytically-sensitive digital melt curve assay method can be used to detect a majority of advanced colorectal adenomas and improve yield over current stool test approaches.

Example 6 – Short DNA as a cancer marker

Free human DNA is present in all human stools and arises from cells shed (exfoliated) from the normal surface (mucosa) of the aero-digestive tract (mouth/throat, lungs, and all digestive organs) and from tumors or other lesions that may be present. It has been generally accepted that "long DNA" in stool reflects that presence of colorectal and other aero-digestive tumors, in that cells exfoliated from cancers do not undergo typical cell death (apoptosis) which would shorten DNA. Specifically, because DNA from apoptotic cells would be broken down to fragment lengths shorter than 100 bp, long DNA was defined as being longer than 100 bp. Indeed, levels of long DNA were elevated in stools from patients with colorectal and other cancers as compared to those from healthy controls (Zou *et al.*, *Cancer Epidemiol. Biomarkers Prev.*, 15:115 (2006); Ahlquist *et al.*, *Gastroenterology*,

119:1219 (2000); and Boynton *et al.*, *Clin. Chem.*, 49:1058 (2003)). As such, long DNA in stool can serve as a marker for colorectal and other tumors.

"Short DNA" (i.e., <100 bp in length), however, was found to be as or more discriminant than long DNA as a tumor marker in stool for detection of both colorectal (Figure 9) and pancreatic (Figure 10) neoplasia.

Briefly, methods and materials similar to those described elsewhere were used to detect short DNA present in stool samples (Zou *et al.*, *Cancer Epidemiol. Biomarkers Prev.*, 15(6):1115 (2006)). Total DNA was extracted by isopropanol precipitation from 19 blinded stool samples: 9 pancreatic adenocarcinoma, and 10 age/gender matched normals. The DNA pellets were taken up in 8 mL of 10-fold diluted TE, pH 8. The Alu sequence consists of conserved regions and variable regions. In the putative consensus Alu sequence, the conserved regions are the 25-bp span between nucleotide positions 23 and 47 and the 16-bp span between nucleotide positions 245 and 260. Although primers can be designed in any part of the Alu sequences, for more effectively amplifying Alu sequences, the PCR primers are preferably completely or partially (at least the 3'-regions of the primers) located in the conserved regions. Primers specific for the human Alu sequences were used to amplify fragments of differing lengths inside Alu repeats. The sequences were as follows:

Amplicon size	Primer Sequences
245 bp	Forward Primer: 5'-ACGCCTGTAATCCCAGCACTT-3' (SEQ ID No:44)
	Reverse Primer: 5'-TCGCCCAGGCTGGAGTGCA-3' (SEQ ID No:45)
130 bp	Forward Primer: 5'-TGGTGAAACCCCGTCTCTAC-3' (SEQ ID No:46)
	Reverse Primer: 5'-CTCACTGCAACCTCCACCTC-3' (SEQ ID No:47)
45 bp	Forward Primer: 5'-TGGTGAAACCCCGTCTCTAC-3' (SEQ ID No:46)
	Reverse Primer: 5'-CGCCCGGCTAATTTTTGTAT-3'(SEQ ID No:48)

Stool DNA was diluted 1:5 with 1x Tris-EDTA buffer (pH 7.5) for PCR amplification. Tris-EDTA buffer–diluted stool DNA (1 μL) was amplified in a total volume of 25 μL containing 1x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 200 nmol/L each primer under the following conditions: 95°C for 3 minutes followed by 40 cycles of 95°C, 60°C, and 72°C for 30 seconds each. A standard curve was created for each plate by

amplifying 10-fold serially diluted human genomic DNA samples (Novagen, Madison, WI). Melting curve analysis was made after each PCR to guarantee that only one product was amplified for all samples.

Amplification was carried out in 96-well plates in an iCycler (Bio-Rad). Each plate consisted of stool DNA samples and multiple positive and negative controls. Each assay was done in duplicate.

The following was performed to compare long DNA (245 bp) and short (45 bp) human DNA in stool for detection of upper and lower GI neoplasms, and to assess the effect of GI tumor site on human DNA levels in stool. Subjects included 33 patients with colorectal cancer, 20 with colorectal adenomas >1 cm, 13 with pancreatic cancer, and 33 colonoscopically-normal controls. Subjects added a preservative buffer to stools at time of collection to prevent post-defecation bacterial metabolism of DNA, and stools were frozen within 8 hours at -80°C. Using a validated quantitative assay for human DNA (Zou *et al.*, *Epidemiol. Biomarkers Prev.*, 15:1115 (2006)), 245 bp and 45 bp Alu sequences were amplified from all stools in blinded fashion. Sensitivities for long and short DNA were based on 97 percent specificity cut-offs.

Age medians were 60, 66, 69, and 62 for colorectal cancer, colorectal adenoma, pancreatic cancer, and control groups, respectively; and male/female distributions were 22/11, 9/11, 9/4, 11/21, respectively. In stools from neoplasm and control groups, amplification products were quantitatively greater for short DNA versus long DNA. Respective sensitivities by long and short DNA were 66 percent and 62 percent with the 29 distal colorectal neoplasms, 46 percent and 46 percent with the 24 proximal colorectal neoplasms, and 15 percent and 31 percent (p=0.16) with the 13 pancreatic cancers. By Wilcoxan Rank-Sum test, effect of neoplasm site on detection rates was significant for both long DNA (p=0.004) and short DNA (p=0.02). Among colorectal neoplasms, respective sensitivities by long and short DNA were 48 percent and 52 percent with lesions <3 cm, 63 percent and 63 percent with those >3 cm, 64 percent and 61 percent with cancers, and 35 percent and 45 percent with adenomas.

These results demonstrate that short and long DNA can be comparably sensitive for stool detection of GI neoplasms. However, detection rates vary with tumor site, being greatest with the most distal lesions and lowest with the most proximal ones. These results were consistent with substantial luminal degradation of DNA exfoliated from more proximal GI neoplasms.

Geneoscopy Exhibit 1067, Page 29

Exact Sciences Ex. 2005, Page 119 of 760

It was also demonstrated that mutant gene markers in stool can be detected to a greater extent if amplicon size is less than 70 bp, consistent with luminal degradation. Thus, short DNA can serve as a marker per se and as the target size for mutation detection.

Example 7 – Use of Fecal Methylated BMP3 as a Neoplasia Marker

Stools from patients with colorectal tumors were found to contain significantly elevated amounts of methylated BMP3 gene copies, but those from normal individuals were found to contain none or only trace amounts. When fecal methylated BMP3 was assayed with an appropriate amplification method, colorectal cancers and premalignant adenomas were specifically detected (Figure 11). Fecal methylated BMP3 detected a higher percentage of proximal colon tumors than distal tumors, so it can be combined with markers for distal colorectal tumors to create complementary marker panels. Fecal methylated BMP3 was very specific with few false-positive reactions.

Similar results can be obtained using other genes and methods such as those described elsewhere (Zou *et al*., *Cancer Epidemiol. Biomarkers Prev*., 16(12):2686 (2007)).

Example 8 – Detecting aero-digestive cancers by stool DNA testing

Tissue samples from patients with confirmed aero-digestive tumors were extracted and sequenced to assess the presence or absence of somatic gene alterations. Germline DNA from the same patients were used as controls. Once an alteration was confirmed, a matched stool sample was tested for that alteration. Two separate methods were utilized to detect the mutation in stool: Allele specific PCR and digital melt curve analysis. For both methods, we focused on amplifying the shortest fragments possible (>100 bp) that have been shown to contain higher levels of the mutant sequence.

Digital Melt Curve (DMC)

We studied 138 patients (69 cases with a GI neoplasm and 69 age/sex-matched asymptomatic controls with normal colonoscopy) by first, identifying a mutation in neoplasm tissue, and then determining if that specific mutation could be detected in stool from that individual. Stools were collected with a stability buffer and frozen at -80C until assayed.

Genes commonly mutated in GI neoplasms (TP53, KRAS, APC, CDH1, CTNNB1, BRAF, SMAD4, and P16) were sequenced from DNA extracted from tumor tissue, to identify a target mutation for each case. Target genes were isolated by hybrid capture (Table 7) and the tissue-confirmed somatic mutations were assayed in stool by the digital melt curve

Geneoscopy Exhibit 1067, Page 30

Exact Sciences Ex. 2005, Page 120 of 760

method, as described in Example 1. Mutations detected in stool were confirmed by sequencing. Assays were performed blinded.

Geneoscopy Exhibit 1067, Page 31

Exact Sciences Ex. 2005, Page 121 of 760

Table 7: Sequence Specific Capture Probes and Primers for AD Cancer Mutation Detection

MUTATION IN TISSUE	CAPTURE PROBE	SEQ ID No.	SENSE PRIMER 1 (5' TO 3')	SEQ ID No.	ANTISENSE PRIMER 2 (5' TO 3')	SEQ ID No.
12487C>CT:167Q>Q/X	ATGGCCATCTACAAGCAGTCATAGCACATGACGGAGGTTGT	49	AGTACTCCCCTGCCCTCAAC	128	CTCACAACCTCCGTCATGTG	169
102447_102450het_delTGGT	AGAGTGAACCATGCAGTGGAAAAGTGGCATTATAAGCCC	50	TTTGAGAGTCGTTCGATTGC	129	CATGGTTTGTCCAGGGCTAT	27
12410G>GA,141C>C/Y	TTTGCCAACTGGCCAAGACCTACCCTGTGCAGCTGTG	51	AGTACTCCCCTGCCCTCAAC	128	CTCCGTCATGTGCTGTGACT	170
102678het_delA	CAGATGCTGATACTTTATTACTTTTGCCACGGAAAGTACT	52	TCCAGGTTCTTCCAGATGCT	130	CACTCAGGCTGGATGAACAA	22
102594_102598het_delAGAGA	AAAGCACCTACTGCTGAAA GAGAGTGGACCTAAGCAAG	53	AGCTCAAACCAAGCGAGAAG	131	AGCATCTGGAAGAACCTGGA	28
102644_102645 het_insG	ATGCTGCAGTTCAGAGGGGTCCAGGTTCTTCCAGATGC	54	GGACCTAAGCAAGCTGCAGTA	132	CACTCAGGCTGGATGAACAA	22
102594_102595het_delAG	TAAAGCACCTACTGCTGAAAAGAGAGTGGACCTAAGCAAG	55	AGCTCAAACCAAGCGAGAAG	131	AGCATCTGGAAGAACCTGGA	28
102106het_delT	CACAGGAAGCAGATTCTGCAATACCCTGCAAATAGCA	56	CAGACGACACAGGAAGCAGA	133	TGCTGGATTTGGTTCTAGGG	171
102442het_delT	TTCAGAGTGAACCATGCAGGGAATGGTAAGTGGCATTAT	57	TTTGAGAGTCGTTCGATTGC	129	CATGGTTTGTCCAGGGCTAT	27
apc 102494C>CT; 1429Q>Q/X	TCCAGATAGCCCTGGATAAACCATGCCACCAAG	58	GTGAACCATGCAGTGGAATG	25	AGCTGTTTGAGGAGGTGGTG	172
apc 102557C>CT; 1450R>R/X	CTCAAACAGCTCAAACCAAGTGAGAAGTACCTAAAAATAAA	59	ACCACCTCCTCAAACAGCTC	134	GCAGCTTGCTTAGGTCCACT	173
apc 102140het_del A	AGCAGAAATAAAAGAAAAGTTGGAACTAGGTCAGCTGA	60	CAGACGACACAGGAAGCAGA	133	TGCTGGATTTGGTTCTAGGG	171
apc 102494C>CT; 1429Q>Q/X	TCCAGATAGCCCTGGATAAACCATGCCACCAAG	58	GTGAACCATGCAGTGGAATG	25	AGCTGTTTGAGGAGGTGGTG	172
apc 102554het_delA	CTCAAACAGCTCAAACCAGCGAGAAGTACCTAAA	61	CATGCCACCAAGCAGAAGTA	21	GCAGCTTGCTTAGGTCCACT	173
tp53 E5 12647A>AG:193H>H/R	TCTGGCCCCTCCTCAGCGTCTTATCCGAGTGGAAG	62	CAGGCCTCTGATTCCTCACT	36	ACACGCAAATTTCCTTCCAC	174
tp53 E5 12742G>GA	CCTATGAGCCGCCTGAGATCTGGTTTGCAACTGGG	63	CATAGTGTGGTGGTGCCCTA	135	AACCACCCTTAACCCCTCCT	175
tp53 E5 12706C>CT:213R>R/X	ATGACAGAAACACTTTTTGACATAGTGTGGTGGTG	64	GTGGAAGGAAATTTGCGTGT	136	CAGTTGCAAACCAGACCTCA	176
tp53 E5 12712A>AG:215S>S/G	GAAACACTTTTCGACATGGTGTGGTGGTGCCCTAT	65	GTGGAAGGAAATTTGCGTGT	136	CAGTTGCAAACCAGACCTCA	176
tp53 E4 12388T>TC:134F>F/L	CTGCCCTCAACAAGATGCTTTGCCAACTGGCCAAG	66	TGTTCACTTGTGCCCTGACT	137	GCAGGTCTTGGCCAGTTG	177
tp53 E3 11606G>GA:125T>T/T	AAGTCTGTGACTTGCACAGTCAGTTGCCCTGAGGG	67	GTCTGGGCTTCTTGCATTCT	138	GCCAGGCATTGAAGTCTCAT	31
tp53 E6 13379C>CT:248R>R/W	GCATGGGCGGCATGAACTGGAGGCCCATCCTCACC	68	TGGCTCTGACTGTACCACCA	139	CCAGTGTGATGATGGTGAGG	178
tp53 I2E3 11326A>AC (splice site)	TCTTTTCACCCATCTACAGTCCCCCTTGCCGTCCC	69	ACCTGGTCCTCTGACTGCTC	140	GGGGACAGCATCAAATCATC	179
tp53 E6 13412G>GT:259D>D/Y	CCATCATCACACTGGAATACTCCAGGTCAGGAGCC	70	CCTCACCATCATCACACTGG	141	GGGTCAGAGGCAAGCAGA	40
tp53 E4 12449G>GT:154G>G/V	ACCCCCGCCCGTCACCCGCGTCC	71	GTGCAGCTGTGGGTTGATT	142	CTCCGTCATGTGCTGTGACT	170
tp53 E7 13872G>GT:298E>E/X	GGAACAGCTTTGAGGTGTGTTTGTGCCTGTCCT	72	GGAAGAGAATCTCCGCAAGA	143	GCTTCTTGTCCTGCTTGCTT	43
APC 102843C>CG:1545S>S/X	TCAGAGCAGCCTAAAGAATGAAATGAAAACCAAGAGAAA	73	ATGCCTCCAGTTCAGGAAAA	144	TTTTTCTGCCTCTTTCTCTTGG	180
tp53 E4 12392G>GT,135C>C/F	CCTCAACAAGATGTTTTTCCAACTGGCCAAGACCT	74	TGCCCTGACTTTCAACTCTGT	145	CTGCACAGGGCAGGTCTT	181
APC 102557C>CT:1450R>R/X	CTCAAACAGCTCAAACCAAGTGAGAAGTACCTAAAAATAAA	59	ACCACCTCCTCAAACAGCTC	134	GCAGCTTGCTTAGGTCCACT	173
tp53 E7 13819G>T:280R>I	CCTGTCCTGGGATAGACCGGCGCAC	75	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182
tp53 I3E4 11326A>AC (splice site)	TCTTTTCACCCATCTACAGTCCCCCTTGCCGTCCC	69	ACCTGGTCCTCTGACTGCTC	140	GGGGACAGCATCAAATCATC	179

tp53 E7 13412G>GT:259D>D/Y	CCATCATCACACTGGAATACTCCAGGTCAGGAGCC	70	CCTCACCATCATCACACTGG	141	GGGTCAGAGGCAAGCAGA	40
tp53 E5 12449G>GT:154G>G/V	ACCCCCGCCCGTCACCCGCGTCC	71	GTGCAGCTGTGGGTTGATT	142	CTCCGTCATGTGCTGTGACT	170
tp53 E8 13872G>GT:298E>E/X	GGAGCCTCACCACTAGCTGCCCCCAGG	76	GGAAGAGAATCTCCGCAAGA	143	GCTTCTTGTCCTGCTTGCTT	43
tp53 E8 13813C>CG:278P>P/R	GTGTTTGTGCCTGTCGTGGGAGAGACCGGCG	77	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182
tp53 E8 13851A>AT:291K>K/X	GGAAGAGAATCTCCGCTAGAAAGGGGAGCCTCA	78	GCGCACAGAGGAAGAGAATC	147	TTCTTGTCCTGCTTGCTTACC	183
smad4 E2 19049G>GA:118A>A/A	GTTAAATATTGTCAGTATGCATTTGACTTAAAATGTGATAG	79	AGGTGGCCTGATCTTCACAA	148	TGGATTCACACAGACACTATCACA	184
tp53 E8 13777G>GA:266G>G/E	TAGTGGTAATCTACTGGAACGGAACAGCTTTGAGGTG	80	TTTCCTTACTGCCTCTTGCTTC	149	CACAAACACGCACCTCAAAG	185
tp53 E6126531T>TC:195I>I/T	CCTCCTCAGCATCTTACCCGAGTGGAAGGAAAT	81	CAGGCCTCTGATTCCTCACT	36	ACACGCAAATTTCCTTCCAC	174
tp53 E7 13379C>CT:248R>R/W	GCATGGGCGGCATGAACTGGAGGCCCATCCTCACC	68	TGGCTCTGACTGTACCACCA	139	CCAGTGTGATGATGGTGAGG	178
tp53 E6 12647A>AG:193H>H/R	TCTGGCCCCTCCTCAGCGTCTTATCCGAGTGGAAG	62	CAGGCCTCTGATTCCTCACT	36	ACACGCAAATTTCCTTCCAC	174
tp53 E6 12712A>AG:215S>S/G	GAAACACTTTTCGACATGGTGTGGTGGTGCCCTAT	65	GTGGAAGGAAATTTGCGTGT	136	CAGTTGCAAACCAGACCTCA	176
tp53 E8 13872G>GT:298E>E/X	GGAGCCTCACCACTAGCTGCCCCCAGG	76	GGAAGAGAATCTCCGCAAGA	143	GCTTCTTGTCCTGCTTGCTT	43
tp53 E7 13370G>GA:245G>G/S	AGTTCCTGCATGGGCAGCATGAACCGGAGGC	82	TGGCTCTGACTGTACCACCA	139	CCAGTGTGATGATGGTGAGG	178
tp53 E4 11580het_delG	CTGGGCTTCTTGCATTCTGGACAGCCAAGTCTGTGA	83	CCCTTCCCAGAAAACCTACC	30	ACTGACCGTGCAAGTCACAG	186
tp53 E5 12524A>AG,179H>H/R	TGCCCCCACCGTGAGCGCTGC	84	TGGCCATCTACAAGCAGTCA	150	CTGCTCACCATCGCTATCTG	187
tp53 E6 12661G>GT,198E>E/X	TCAGCATCTTATCCGAGTGTAAGGAAATTTGCGTGTGGA	85	CAGGCCTCTGATTCCTCACT	36	CCAAATACTCCACACGCAAA	188
tp53 E8 13872G>GT:298E>E/X	GGAGCCTCACCACTAGCTGCCCCCAGG	76	GGAAGAGAATCTCCGCAAGA	143	GCTTCTTGTCCTGCTTGCTT	43
apc 102494C>CT; 1429Q>Q/X	TCCAGATAGCCCTGGATAAACCATGCCACCAAG	58	CAGGAGACCCCACTCATGTT	19	TGGCAAAATGTAATAAAGTATCAGC	20
apc 102557C>CT; 1450R>R/X	CTCAAACAGCTCAAACCAAGTGAGAAGTACCTAAAAATAAA	59	CAGGAGACCCCACTCATGTT	19	TGGCAAAATGTAATAAAGTATCAGC	20
apc 102140het_delA	AGCAGAAATAAAAGAAAAGTTGGAACTAGGTCAGCTGA	60	TTCATTATCATCTTTGTCATCAGC	15	CGCTCCTGAAGAAAATTCAA	16
apc 102494C>CT; 1429Q>Q/X	TCCAGATAGCCCTGGATAAACCATGCCACCAAG	58	CAGGAGACCCCACTCATGTT	19	TGGCAAAATGTAATAAAGTATCAGC	20
apc 102134G>GT; 1309E>E/X	TGCAAATAGCAGAAATAAAATAAAAGATTGGAACTAGGTCA	86	TTCATTATCATCTTTGTCATCAGC	15	CGCTCCTGAAGAAAATTCAA	16
apc 102554het_delA	CTCAAACAGCTCAAACCAGCGAGAAGTACCTAAA	61	CAGGAGACCCCACTCATGTT	19	TGGCAAAATGTAATAAAGTATCAGC	20
apc 102852het_insA	CTAAAGAATCAAATGAAAAACCAAGAGAAAGAGGCAGAA	87	GAGCCTCGATGAGCCATTTA	23	TCAATATCATCATCATCTGAATCATC	24
kras 5571G>GA; 12G>G/D	GTGGTAGTTGGAGCTGATGGCGTAGGCAAGAGT	88	AGGCCTGCTGAAAATGACTG	2	TTGTTGGATCATATTCGTCCAC	3
tp53 E4 12392G>GA; 135C>C/Y	CCTCAACAAGATGTTTTACCAACTGGCCAAGACCT	89	TGTTCACTTGTGCCCTGACT	137	GCAGGTCTTGGCCAGTTG	177
tp53 E5 12655C>CT; 196R>R/X	CCTCCTCAGCATCTTATCTGAGTGGAAGGAAATTTGC	90	CAGGCCTCTGATTCCTCACT	36	ACACGCAAATTTCCTTCCAC	174
tp53 E6 13350G>GA; 238C>C/Y	TCCACTACAACTACATGTATAACAGTTCCTGCATGGG	91	TGGCTCTGACTGTACCACCA	139	CCAGTGTGATGATGGTGAGG	178
tp53 E6 13420G>GA	CACTGGAAGACTCCAGATCAGGAGCCACTTGCC	92	CCTCACCATCATCACACTGG	141	GGGTCAGAGGCAAGCAGA	40
tp53 E5 12712A>AG; 215S>S/G	GAAACACTTTTCGACATGGTGTGGTGGTGCCCTAT	65	GTGGAAGGAAATTTGCGTGT	136	CAGTTGCAAACCAGACCTCA	176
kras 5571G>GA; 12G>G/D	GTGGTAGTTGGAGCTGATGGCGTAGGCAAGAGT	88	AGGCCTGCTGAAAATGACTG	2	TTGTTGGATCATATTCGTCCAC	3
p16 (ink4a) E1 19638A>AT	GGAGAGGGGGAGTGCAGGCAGCGGG	93	AGCCAGTCAGCCGAAGG	151	GAGGGGCTGGCTGGTC	189
p16 (ink4a) E2 23353G>GT; 447D>D/Y	CCCAACTGCGCCTACCCCGCCACTC	94	CACCCTGGCTCTGACCAT	152	GGGTCGGGTGAGAGTGG	190
p16 (ink4a) E1 19638A>AT	GGAGAGGGGGAGTGCAGGCAGCGGG	93	AGCCAGTCAGCCGAAGG	151	GAGGGGCTGGCTGGTC	189

Geneoscopy Exhibit 1067, Page 33

Exact Sciences Ex. 2005, Page 123 of 760

p16 (ink4a) E2 23402het_delT	GCCCGGCGAGGGCTCCTGGACACGCTG	95	GACCCCGCCACTCTCAC	153	CAGCTCCTCAGCCAGGTC	191
p16 (ink4a) E2 23403C>CA; 484F>F/	GCCCGGCGAGGGCTTACTGGACACGCTGGT	96	GACCCCGCCACTCTCAC	153	CAGCTCCTCAGCCAGGTC	191
ctnnb1 25541het_delT	CAATGGGTCATATCACAGATTCTTTTTTTTAAATTAAAGTAACA	97	ATATTTCAATGGGTCATATCACAG	154	TCAAATCAGCTATAAATACGAAACA	192
cdh1 E9 76435het_delA	TCTTATCTCAAAAGAACAACAAAAAAGAGGAATCCTTTAG	98	GCCATGATCGCTCAAATACA	155	TCTCAGGGGGCTAAAGGATT	193
cdh1 E1 743_744het_insAGCCCTGCGCCCA	GCGCCCAGCCCTGCGCCCATTCCTC	99	ACTTGCGAGGGACGCATT	156	GAAGAAGGGAAGCGGTGAC	194
cdh1 E13 86853_86854het_insA	AAGTAAGTCCAGCTGGCAAAGTGACTCAGCCTTTGACTT	100	CATTCTGGGGATTCTTGGAG	157	GGAAATAAACCTCCTCCATTTTT	195
cdh1 E14 91472C>CT; 751N>N/N	AGGATGACACCCGGGACAATGTTTATTACTATGATGAAG	101	CTGTTTCTTCGGAGGAGAGC	158	CCGCCTCCTTCTTCATCATA	196
cdh1 E15 92868_92896het_delTTGACTTGA GCCAGCTGCACAGGGGCCTG	TTTTTTCTCCAAAGGACTGACGCTCGGCCTGAAGTG	102	TTCCTACTCTTCATTGTACTTCAACC	159	TGCAACGTCGTTACGAGTCA	197
cdh1 E1 736_742het_delTGCGCCC	AGCCCTGCGCCCCTTCCTCTCCCG	105	ACTTGCGAGGGACGCATT	156	GAAGAAGGGAAGCGGTGAC	194
p16 (ink4a) E1 196368A>AT	GGAGAGGGGGAGTGCAGGCAGCGGG	93	AGCCAGTCAGCCGAAGG	151	GAGGGGCTGGCTGGTC	189
tp53 E4 12365A>AG; 126Y>Y/C	TTCCTCTTCCTACAGTGCTCCCCTGCCCTCAAC	106	CACTTGTGCCCTGACTTTCA	33	GCCAGTTGGCAAAACATCT	200
tp53 E4 12548G>GA	TGCTCAGATAGCGATGATGAGCAGCTGGGGCTG	107	CACATGACGGAGGTTGTGAG	162	AACCAGCCCTGTCGTCTCT	34
p16 (ink4a) E1 19810T>TG; 491I>I/S	GGTCGGAGGCCGAGCCAGGTGGGTAGA	108	TTCCAATTCCCCTGCAAA	163	CCCAACGCACCGAATAGT	201
tp53 E7 13757G>GA	GCTTCTCTTTTCCTATCCTAAGTAGTGGTAATCTACTGG	109	GGGACAGGTAGGACCTGATTT	164	AGCTGTTCCGTCCCAGTAGA	202
tp53 E7 13815G>GC; 279G>G/R	TTGTGCCTGTCCTCGGAGAGACCGGCG	110	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182
tp53 E7 13816G>GA; 279G>G/E	TGTGCCTGTCCTGAGAGAGACCGGCGC	111	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182
tp53 E5 12365A>AC, 126Y>Y/S	TTCCTCTTCCTACAGTCCTCCCCTGCCCTCAAC	112	CACTTGTGCCCTGACTTTCA	33	GCCAGTTGGCAAAACATCT	200
tp53 E5 12491A>AT,168H>H/L	TCTACAAGCAGTCACAGCTCATGACGGAGGTTGTGGA	113	TGGCCATCTACAAGCAGTCA	150	CTGCTCACCATCGCTATCTG	187
		113	TGGCCATCTACAAGCAGTCA	150	TCACCATCGCTATCTGAGCA	203
		113	TGGCCATCTACAAGCAGTCA	150	AACCAGCCCTGTCGTCTCT	34
kras 5570G>GC;12G>G/R	GTGGTAGTTGGAGCTGATGGCGTAGGCAAGAGT	88	AGGCCTGCTGAAAATGACTG	2	TTGTTGGATCATATTCGTCCAC	3
tp53 E7 13370G>GA;245G>G/S	AGTTCCTGCATGGGCAGCATGAACCGGAGGC	82	TGGCTCTGACTGTACCACCA	139	CCAGTGTGATGATGGTGAGG	178
apc 102864_102865het_delAG	AAATGAAAACCAAGAGAAAGGCAGAAAAAACTATTGATTC	114	TGACAATGGGAATGAAACAGA	165	GGTCCTTTTCAGAATCAATAGTTTT	204
tp53 E5 12386T>TC,133M>M/T	CCTGCCCTCAACAAGATGTTTTGCCAACTGGCC	115	TGTTCACTTGTGCCCTGACT	137	GCAGGTCTTGGCCAGTTG	177
cdh1 E15 93059G>GA	GCTCATCTCTAAGCTCAGGAAGAGTTGTGTCAAAAATGAGA	116	CCAAAGCATGGCTCATCTCTA	205	CTCAGGCAAGCTGAAAACAT	206
tp53 E8 13798G>GA;273R>R/H	CGGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGG	117	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182
p53 E6, 12698_12701het_delAC (1 or 2 AC repeats)	TGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAG	118	GTGGAAGGAAATTTGCGTGT	136	CAGTTGCAAACCAGACCTCA	176
P53 E8, 13824C>CT,282R>R/W	TGTCCTGGGAGAGACTGGCGCACAGAGGAAGAGAAT	119	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182

Geneoscopy Exhibit 1067, Page 34

Exact Sciences Ex. 2005, Page 124 of 760

APC 102151G>GA,1314R>R/R	AAGAAAAGATTGGAACTAGATCAGCTGAAGATCCTGTG	120	CAGACGACACAGGAAGCAGA	133	GTGACACTGCTGGAACTTCG	207
P53 E5 12457 G>G/T	CGCCCGGCACCCGCTTCCGCGCCATGGCCA	121	GTGCAGCTGTGGGTTGATT	142	CTCCGTCATGTGCTGTGACT	170
p53 E8 13812C>CG,278P>P/A	GTGTTTGTGCCTGTGCTGGGAGAGACCGGCG	122	CTACTGGGACGGAACAGCTT	146	GCGGAGATTCTCTTCCTCTG	182
APC 102686het_delA	AGGTTCTTCCAGATGCTGATACTTTATTACATTTTGC	123	CTGCAGTTCAGAGGGTCCAG	210	CACTCAGGCTGGATGAACAA	22
APC het_delAG between 102594_102603 (1 of 5 AG repeats)	GCGAGAAGTACCTAAAAATAAAGCACCTACTGCTGAA	124	AGCTCAAACCAAGCGAGAAG	131	AGCATCTGGAAGAACCTGGA	28
APC 102240C>CA,1344S>S/X	CAGGGTTCTAGTTTATCTTAAGAATCAGCCAGGCACA	125	CCCTAGAACCAAATCCAGCA	166	TGTCTGAGCACCACTTTTGG	208
102676_102680delACATT	CCAGATGCTGATACTTTATTTTGCCACGGAAAGTACTC	126	CTGCAGTTCAGAGGGTCCAG	167	CACTCAGGCTGGATGAACAA	22
12487C>CT:1672Q>Q/X	ATGGCCATCTACAAGCAGTCATAGCACATGACGGAGGTTGT	49	AGTACTCCCCTGCCCTCAAC	128	CTCACAACCTCCGTCATGTG	169
102447_102450het_delTGGT	AGAGTGAACCATGCAGTGGAAAAGTGGCATTATAAGCCC	50	TTTGAGAGTCGTTCGATTGC	129	CATGGTTTGTCCAGGGCTAT	27
12410G>GA,141C>C/Y	TTTGCCAACTGGCCAAGACCTACCCTGTGCAGCTGTG	51	AGTACTCCCCTGCCCTCAAC	128	CTCCGTCATGTGCTGTGACT	170
102678het_delA	CAGATGCTGATACTTTATTACTTTTGCCACGGAAAGTACT	52	TCCAGGTTCTTCCAGATGCT	130	CACTCAGGCTGGATGAACAA	22
102594_102598het_delAGAGA	AAAGCACCTACTGCTGAAAGAGAGTGGACCTAAGCAAG	127	AGCTCAAACCAAGCGAGAAG	131	AGCATCTGGAAGAACCTGGA	28
102776A>AT:1523R>R/X	ATGACAATGGGAATGAAACAGAATCAGAGCAGCCTAAAG	14	TTTGCCACGGAAAGTACTCC	168	TTTCCTGAACTGGAGGCATT	209
102644_102645 het_insG	ATGCTGCAGTTCAGAGGGGTCCAGGTTCTTCCAGATGC	54	GGACCTAAGCAAGCTGCAGTA	132	CACTCAGGCTGGATGAACAA	22
102594_102595het_delAG	TAAAGCACCTACTGCTGAAAAGAGAGTGGACCTAAGCAAG	55	AGCTCAAACCAAGCGAGAAG	131	AGCATCTGGAAGAACCTGGA	28
102106het_delT	CACAGGAAGCAGATTCTGCAATACCCTGCAAATAGCA	56	CAGACGACACAGGAAGCAGA	133	TGCTGGATTTGGTTCTAGGG	171
102442het_delT	TTCAGAGTGAACCATGCAGGGAATGGTAAGTGGCATTAT	57	TTTGAGAGTCGTTCGATTGC	129	CATGGTTTGTCCAGGGCTAT	27
apc 102494C>CT; 1429Q>Q/X	TCCAGATAGCCCTGGATAAACCATGCCACCAAG	58	GTGAACCATGCAGTGGAATG	25	AGCTGTTTGAGGAGGTGGTG	172
apc 102140het_delA	AGCAGAAATAAAAGAAAAGTTGGAACTAGGTCAGCTGA	60	CAGACGACACAGGAAGCAGA	133	TGCTGGATTTGGTTCTAGGG	171
apc 102554het_delA	CTCAAACAGCTCAAACCAGCGAAGTACCTAAA	61	CATGCCACCAAGCAGAAGTA	21	GCAGCTTGCTTAGGTCCACT	173

Geneoscopy Exhibit 1067, Page 35

Exact Sciences Ex. 2005, Page 125 of 760

Target mutations were not detected in control stools. Target mutations were detected in stools from 68% (47/69) of patients with a GI neoplasm. Specifically, target mutations were detected in stools from 71% (36/51) of patients with cancer [40% (2/5) with oropharyngeal , 65% (11/17) with esophageal, 100% (4/4) with gastric, 55% (6/11) with pancreatic, 75% (3/4) with biliary or gallbladder, and 100% (10/10) with colorectal] and from 61%(11/18) with precancers [100% (2/2) with pancreatic intraductular papillary mucinous neoplasia and 56% (9/16) with colorectal advanced adenoma]. Mutant copies in genes recovered from stool averaged 0.4% (range 0.05-13.4%) for supracolonic and 1.4% (0.1-15.6%) for colorectal neoplasms, p=0.004 (Table 8).

Table 8: Digital Melt Curve Detection of Validated Mutations in AD Cancer Patient Stool

#	ID	Site	Age	Gender	Tissue Mutations			Stool Detection	Mutation Frequency %	Normal Control
1	1163	Head/Neck (pharynx)	73	M	tp53			YES	0.8	Neg
2	1250	Head/Neck (pharynx)	49	M	tp53			NO		Neg
3	1295	Head/Neck (pharynx)	47	F	tp53			NO		Neg
4	1391	Head/Neck	65	M	tp53	TP53		NO (both)		Neg
5	1427	Head/Neck	60	M	tp53	tp53		YES (p53-1), No (p53-2)	0.05	Neg
1	745	Esophagus	84	F	tp53			YES	0.4	Neg
2	769	Esophagus	56	F	tp53			YES	0.4	Neg
3	782	Esophagus	55	M	tp53			NO		Neg
4	789	Esophagus	61	M	tp53			YES	1.6	Neg
5	819	Esophagus	53	M	tp53			YES	0.2	Neg
6	873	Esophagus	61	M	tp53			YES	0.2	Neg
7	906	Esophagus	55	M	APC			YES	0.8	Neg
8	1049	Esophagus	57	M	tp53			NO		Neg
9	1064	Esophagus	72	F	tp53			NO		Neg
10	1067	Esophagus	72	M	tp53			YES	0.7	Neg
11	1103	Esophagus	78	M	tp53			NO		Neg

Geneoscopy Exhibit 1067, Page 36

Exact Sciences Ex. 2005, Page 126 of 760

12	1199	Esophagus	66	M	tp53			YES	0.5	Neg
13	1307	Esophagus	51	M	tp53			NO		Neg
14	1373	Esophagus	76	M	tp53			YES	0.5	Neg
15	1414	Esophagus	66	M	tp53			YES	0.1	Neg
16	1448	Esophagus	82	M	tp53			NO		Neg
17	1072	Esophagus			tp53			YES	0.4	Neg
1	798	Stomach	81	M	cdh1			YES	13.2	Neg
3	1221	Stomach	55	M		cdh1	cdh1	YES (both)	8, 1.3	Neg
4	1224	Stomach	75	F	smad4	cdh1		YES (smad4), NO (CDH1)	0.2	Neg
5	1402	Stomach	56	M	APC	tp53		YES (p53)	0.1	Neg
1	848	Gall Bladder	67	M	tp53			YES	0.1	Neg
2	1315	Gall Bladder	57	F	tp53			YES	1.4	Neg
1	1043	Bile Duct	51	F	APC			NO		Neg
2	1554	Bile Duct	77	M	cdh1			YES	13.4	Neg
1	757	Pancceatic cancer in situ	78	M	k-ras			YES	0.2	Neg
2	1349	Pancreatic cancer in situ	64	M	k-ras			YES	0.2	Neg
1	839	Pancreas	69	F	tp53			YES	0.2	Neg
2	1204	Pancreas	65	F		p16		No		Neg
3	1253	Pancreas	63	F	K-ras		tp53	YES (K-ras), No (p53)	2	Neg
4	1400	Pancreas	71	F	tp53	k-ras		NO (both)		Neg
5	1547	Pancreas	77	F	tp53			NO		Neg
6	1217	Pancreas			K-ras			NO		Neg
7	1073	Pancreas			K-ras			NO		Neg
8	532	Pancreas			K-ras			YES	1	Neg
9	1592	Pancreas			K-ras	P53		YES (both)	0.3	Neg
10	1695	Pancreas			K-ras			YES	0.2	Neg
11	1058	Pancreas			K-ras	P53	APC	YES (K-ras)	0.2	Neg
1	438	Colorectal Cancer	78	F		APC		YES	1.2	Neg
2	446	Colorectal Cancer	74	M	BRAF			YES	0.4	Neg
3	529	Colorectal Cancer	46	M	K-RAS			YES	1	Neg
4	489	Colorectal Cancer	73	M	K-RAS			YES	2.6	Neg
5	549	Colorectal Cancer	79	M	BRAF			YES	1.6	Neg
6	551	Colorectal Cancer	69	M	K-RAS			YES	5.8	Neg
7	584	Colorectal Cancer	68	M	K-RAS			YES	1.4	Neg
8	894	Colorectal Cancer	57	M	P53	APC		YES (p53, APC)	1.6, 5	Neg
9	998	Colorectal Cancer	45	F	APC	K-RAS		YES (K-ras, APC)	0.6, 0.8	Neg
10	1009	Colorectal Cancer	65	F	P53			YES	12.9	Neg
1	513	Colorectal Adenoma	65	F	APC			YES	0.1	Neg
2	546	Colorectal Adenoma	61	M	APC			NO		Neg
3	547	Colorectal Adenoma	52	F	APC			NO		Neg
4	568	Colorectal Adenoma	52	M	APC			YES	7.8	Neg
5	578	Colorectal Adenoma	71	F	APC			NO		Neg
6	590	Colorectal Adenoma	54	F	APC			YES	3.2	Neg
7	701	Colorectal Adenoma	72	F	APC			NO		Neg
8	855	Colorectal Adenoma	75	M	K-RAS			YES	0.4	Neg
9	860	Colorectal Adenoma	53	M	APC			YES	15.6	Neg
10	900	Colorectal Adenoma	64	F	APC	K-RAS		NO (both)		Neg
11	962	Colorectal Adenoma	56	M	K-RAS			YES	1	Neg
12	965	Colorectal Adenoma	82	M	APC	K-RAS		NO (both)		Neg
13	991	Colorectal Adenoma	79	M	APC	K-RAS		YES (K-ras), No (APC)	0.2	Neg
14	1135	Colorectal Adenoma	59	M	K-RAS			YES	13	Neg
15	1231	Colorectal Adenoma	50	M	APC			NO		Neg
16	1559	Colorectal Adenoma			K-RAS			YES	1	Neg

We also performed an initial pilot study with 10 stool samples from patients with confirmed bile duct cancers to determine if DMC technology could detect mutations in k-ras, a well characterized gene known be mutated in this population. K-ras mutations were detected in stools for 3/10 or 4/10 bile duct cancers (depending on mutation score of 5 or 3, respectively) (Table 9). As K-ras is mutant in 30-40% of bile duct cancers, these results indicate that the detection assay is picking up the appropriate proportion of cancer samples.

Geneoscopy Exhibit 1067, Page 37

Exact Sciences Ex. 2005, Page 127 of 760

Table 9. K-ras mutation scores for patients with bile duct cancer.

			Mutation Detected	
Sample #	Pathology	K-ras Mutation Score	A	B
520	BD Cancer	0		
528	BD Cancer	0		
559	BD Cancer	0		
558	BD Cancer	1	Codon 12 GAT	
515	BD Cancer	2	Codon 12 GAT	
543	BD Cancer	2	Codon 13 GAC	
806	BD Cancer	**3**	Codon 12 TGT	
539	BD Cancer	**5**	Codon 12 GAT	Codon 13 GGA
512	BD Cancer	**6**	Codon 12 GAT	Codon 12 GAT
725	BD Cancer	**25**	Codon 13 GAC	Codon 12 GAT; Codon 12 GTT

Allele Specific PCR

The allele specific-PCR assay was a modified version of a previously published method (*e.g.*, Cha et al., Mismatch Amplification Mutation Assay (MAMA): Application to the c-H-ras Gene PCR Methods and Applications, 2:14-20 (1992) Cold Spring Harbor Laboratory). TP53 gene fragments were captured from stool DNA samples with probes specific to mutations identified in the matched tissue (Table 7). Copy numbers were assessed by qPCR. Samples were adjusted to 10,000 fragments each and amplified with allele specific primer sets.

Sample	F Primer	R Primer
A745	GACAGAAACACTTTAT (SEQ ID No:211)	CGGCTCATAGGG (SEQ ID No:217)
A848	ACACTTTTCGACAAG (SEQ ID No:212)	AAACCAGACCTCAG (SEQ ID No:218)
A789	CCTCAACAAGATAC (SEQ ID No:213)	CAGCTGCACAGG (SEQ ID No:219)
A782	GCCGCCTGAAA (SEQ ID No:214)	AGACCCCAGTTGC (SEQ ID No:220)
A873	GCGGCATGAAAT (SEQ ID No:215)	TTCCAGTGTGATGAT (SEQ ID No:221)
A769	CCCCTCCTCAGAG (SEQ ID No:216)	CTTCCACTCGGATAA (SEQ ID No:222)

The forward primer in each case is specific for each TP53 mutation.

Esophagus and Stomach

Targeting mutations found in esophageal cancers or those from gastroesophageal junction (on p53, APC, or K-ras), the same mutation was detected by allele-specific PCR in matched stools from five of five (100%) cancers but in none of the controls (Table 10). The

Geneoscopy Exhibit 1067, Page 38

threshold cycle (Ct), designates the PCR cycle at which the product enters the exponential phase of amplification.

Gallbladder

Targeting a mutation confirmed in a gallbladder cancer, the same mutation was found in the matched stool from that patient using allele-specific PCR (Table 10).

Table 10. Quantitative Mutant Allele Specific-PCR Results for Matched Aero-digestive Cancers

Sample		Gene	# fragments		Ct
A769	esophageal/gastric cancer	p53	10K		71
N	normal	p53	10K		>80
A782	esophageal/gastric cancer	p53	10K		38.8
N	normal	p53	10K		44.1
A745	esophageal/gastric cancer	p53	30K		42.5
N	normal	p53	30K		45.6
A873	esophageal/gastric cancer	p53	30K		37.8
N	normal	p53	30K		40.4
A848	gall bladder cancer	p53	10K		22.4
N	normal	p53	10K		36.3
A789	esophageal/gastric cancer	p53	10K		25.9
N	normal	p53	10K		28.7

Example 9 – Candidate Stool Polypeptide Markers Identified for Colorectal Cancer and Precancerous Adenomas

The following list of polypeptides were identified by a statistical analysis model using all data generated from mass spectral of fecal protein extracts: β2-macroglobulin, compliment C3 protein, serotransferrin, haptoglobin, carbonic anhydrase 1, xaa-pro dipeptidase, leukocyte elastase inhibitor, hemoglobin, glucose-6-phosphate, and catalase. This list of polypeptides is in order of difference from normal. Thus, the mean spectral abundance for β2-macroglobulin is most different from normal for cancer and adenoma.

Geneoscopy Exhibit 1067, Page 39

Exact Sciences Ex. 2005, Page 129 of 760

The statistical significance of relative polypeptide abundance between normal, adenoma, and colorectal cancer (CRC) was obtained using normalized spectral count data from a zero inflated poisson regression model as an offset term in the protein specific differential expression analysis. The differential expression analysis also incorporated the zero inflated poisson regression model. Polypeptides were then ranked according to their statistical significance and whether the expression profile followed the clinically relevant pattern of Normal < Adenoma < CRC. Using a rule that a positive test required that any three of top six markers be positive, the sensitivity and specificity of this panel were both 100% in a training set.

The listed polypeptides can be used individually or in any combination to detect colorectal cancer or precancerous adenomas.

Example 10 – Identification of polypeptide markers for pancreatic cancer

Potential polypeptide markers for pancreatic cancer prediction were identified. Utilizing a Scaffold (Proteome Software) side-by-side comparison of spectral abundances, ratios of the spectral counts of carboxypeptidase B (CBPB1_HUMAN) and Carboxypeptidase A1 (CBPA1_HUMAN) were compared. A value for carboxypeptidase B/A1 of 0.7 or higher was predictive of pancreatic cancer (in normal stools, an average ratio of 2:3 B/A1 was observed). Specificity for training data was 100% with a sensitivity of 88%, while sensitivity from a validation set was 82% at the same specificity.

Example 11 – Use of Fecal Methylated ALX4 as a Neoplasia Marker

Stools from patients with colorectal tumors were found to contain significantly elevated amounts of methylated ALX4 gene copies, but those from normal individuals were found to contain none or only trace amounts. When fecal methylated ALX4 was assayed with an appropriate amplification method, colorectal cancers and premalignant adenomas were specifically detected. At 90% specificity, fecal methylated ALX4 detected 59% colorectal cancer and 54% premalignant adenomas, allowing for the detection of both colorectal cancer and premalignant adenomas.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not

Geneoscopy Exhibit 1067, Page 40

Exact Sciences Ex. 2005, Page 130 of 760

limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Geneoscopy Exhibit 1067, Page 41

Exact Sciences Ex. 2005, Page 131 of 760

WHAT IS CLAIMED IS:

1. A method of detecting pancreatic cancer in a mammal, wherein said method comprises determining the ratio of an elastase 3A polypeptide to a pancreatic alpha-amylase polypeptide present within a stool sample, wherein the presence of a ratio greater than about 0.5 indicates that said mammal has said pancreatic cancer, and wherein the presence of a ratio less than about 0.5 indicates that said mammal does not have said pancreatic cancer.

2. A method of detecting pancreatic cancer in a mammal, said method comprising determining the level of an elastase 3A polypeptide in a stool sample from said mammal, wherein the presence of an increased level of an elastase 3A polypeptide when compared to a normal control level is indicative of pancreatic cancer in said mammal.

3. A method of detecting pancreatic cancer in a mammal, said method comprising determining the level of a carboxypeptidase B polypeptide in a stool sample from said mammal, wherein the presence of an increased level of a carboxypeptidase B polypeptide when compared to a normal control level is indicative of pancreatic cancer in said mammal.

4. A method of detecting pancreatic cancer in a mammal, said method comprising determining whether or not a stool sample from said mammal comprises a ratio of a carboxypeptidase B polypeptide to a carboxypeptidase A2 polypeptide that is greater than about 0.5, wherein the presence of said ratio greater than 0.5 indicates that said mammal has said pancreatic cancer.

5. A method of detecting cancer or pre-cancer in a mammal, said method comprising determining whether or not a stool sample from said mammal comprises an increase in the number of DNA fragments less than 200 base pairs in length as compared to a normal control, wherein the presence of said increase number of DNA fragments less than 200 base pairs in length indicates that said mammal has said cancer or pre-cancer.

6. The method of claim 5, wherein said DNA fragments are less than 70 base pairs in length.

Geneoscopy Exhibit 1067, Page 42

Exact Sciences Ex. 2005, Page 132 of 760

7. A method of detecting colorectal cancer or pre-cancer in a mammal, said method comprising determining whether or not a stool sample from said mammal comprises an elevated K-ras mutation score, an elevated BMP3 methylation status, and an elevated level of human DNA as compared to a normal control, wherein the presence of said elevated K-ras mutation score, said elevated BMP3 methylation status, and said elevated level of human DNA level indicates that said mammal has said colorectal cancer or pre-cancer.

8. The method of claim 7, wherein said K-ras mutation score is measured by digital melt curve analysis.

9. The method of claim 7, wherein said K-ras mutation score is measured by quantitative allele-specific PCR.

10. A method of detecting aero-digestive cancer or pre-cancer in a mammal, said method comprising determining whether or not a stool sample from said mammal comprises an elevated K-ras mutation score, an elevated BMP3 methylation status, and an elevated level of human DNA as compared to a normal control, wherein the presence of said elevated K-ras mutation score, said elevated BMP3 methylation status, and said elevated level of human DNA level indicates that said mammal has said aero-digestive cancer or pre-cancer.

11. The method of claim 10, wherein said K-ras mutation score is measured by digital melt curve analysis.

12. The method of claim 10, wherein said K-ras mutation score is measured by quantitative allele-specific PCR.

13. The method of claim 10, wherein said method further comprises determining whether or not a stool sample from said mammal comprises an elevated APC mutation score.

14. The method of claim 13, wherein said APC mutation score can be measured by digital melt curve analysis.

15. A method of detecting aero-digestive cancer or pre-cancer in a mammal, said method comprising determining whether or not said mammal comprises at least one

Geneoscopy Exhibit 1067, Page 43

Exact Sciences Ex. 2005, Page 133 of 760

mutation in six nucleic acids selected from a group consisting of a p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF, wherein the presence of at least one mutation in each of said six nucleic acids indicates that said mammal has said aero-digestive cancer or pre-cancer.

16. The method of claim 15, wherein said method further comprises determining whether or not a stool sample from said mammal comprises an elevated level of a carboxypeptidase B polypeptide as compared to a normal control, wherein the presence of said elevated level of a carboxypeptidase B polypeptide indicates that said mammal has said aero-digestive cancer or pre-cancer in said mammal.

17. The method of claim 15, wherein said method further comprises determining whether or not a stool sample from said mammal comprises an elevated amount of DNA fragments less than 70 base pairs in length as compared to a normal control, wherein the presence of said elevated amount of DNA fragments less than 70 base pairs in length indicates that said mammal has said aero-digestive cancer or pre-cancer.

18. The method of claim 15, wherein said method further comprises determining whether or not a stool sample from said mammal comprises an elevated amount of DNA fragments greater than 100 base pairs in length as compared to normal controls, wherein the presence of said elevated amount of DNA fragments greater than 100 base pairs in length indicates that said mammal has said aero-digestive cancer or pre-cancer.

19. The method of claim 15, wherein said method further comprises determining whether or not a stool sample from said mammal comprises an elevated BMP3 methylation status, wherein the presence of said elevated methylation status indicates that said mammal has said aero-digestive cancer or pre-cancer.

20. The method of claim 15, wherein said determining step comprises digital melt curve analysis.

21. A method of detecting aero-digestive cancer or pre-cancer in a mammal, said method comprising determining the number of mutations in a p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF nucleic acid, present in a stool sample from said mammal, wherein the presence of a mutation in each of said nucleic acids indicates the presence of said aero-digestive cancer or pre-cancer in said mammal.

Geneoscopy Exhibit 1067, Page 44

Exact Sciences Ex. 2005, Page 134 of 760

22. A method of detecting aero-digestive cancer in a mammal, said method comprising determining whether or not the methylation status of an ALX4 nucleic acid in a stool sample from said mammal is elevated when compared to a normal control level, wherein said elevated methylation status indicatives the presence of said aero-digestive cancer in said mammal.

23. A method of diagnosing pancreatic cancer in a mammal, said method comprising obtaining a stool sample from said mammal, determining the ratio of an elastase 3A polypeptide to a pancreatic alpha-amylase polypeptide present within said stool sample, and communicating a diagnosis of pancreatic cancer if said ratio is greater than about 0.5, thereby diagnosing said mammal with pancreatic cancer.

24. A method of diagnosing a mammal with pancreatic cancer, said method comprising obtaining a stool sample from said mammal, detecting mutations in a p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF nucleic acid within said sample, determining the ratio of a carboxypeptidase B polypeptide to a carboxypeptidase A2 polypeptide present within said sample, and communicating a diagnosis of pancreatic cancer or pre-cancer if said mutations are detected in each of said nucleic acids and said ratio of carboxypeptidase B polypeptide to carboxypeptidase A2 polypeptide is greater than 0.5, thereby diagnosing said mammal.

25. A method of diagnosing a mammal with colorectal cancer, said method comprising obtaining a stool sample from said mammal, detecting mutations in a group of nucleic acids consisting of a p16, p53, k-ras, APC, SMAD4, EGFR, CTNNB1, and BRAF nucleic acid within DNA present in said sample, measuring the level of a serotransferrin polypeptide present within said sample, and communicating a diagnosis of colorectal cancer or pre-cancer if said mutations are detected in each of said nucleic acids and said level of serotransferrin is elevated as compared to a reference level, thereby diagnosing said mammal.

Geneoscopy Exhibit 1067, Page 45

Exact Sciences Ex. 2005, Page 135 of 760

DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: PCT/US09/33793 Document Date: 02/11/2009

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Form Revision Date: February 8, 2006

Figure 1

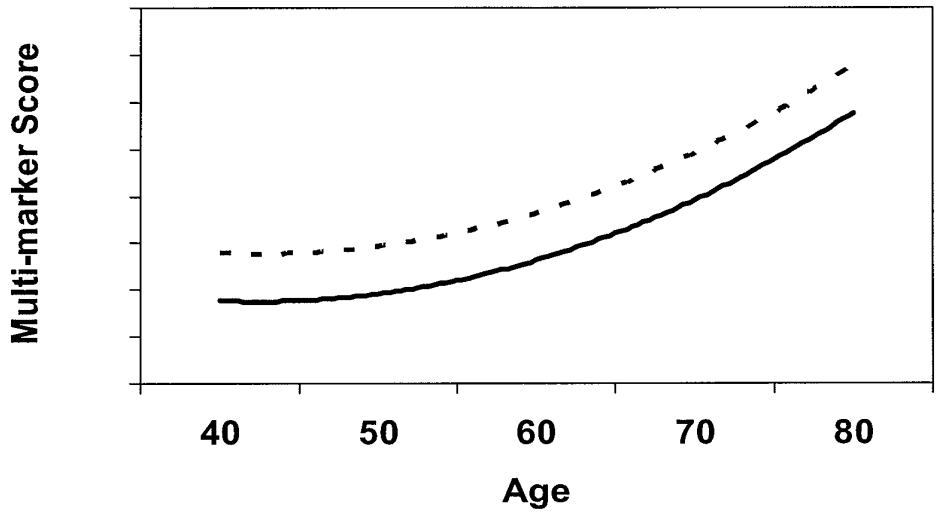


FIGURE 2

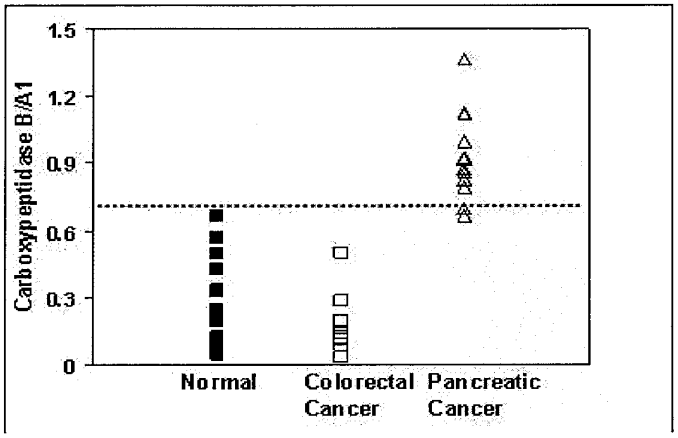

Carboxypeptidase B/A1
1.5
1.2
0.9
0.6
0.3
0
Normal
Colorectal Cancer
Pancreatic Cancer

FIGURE 3

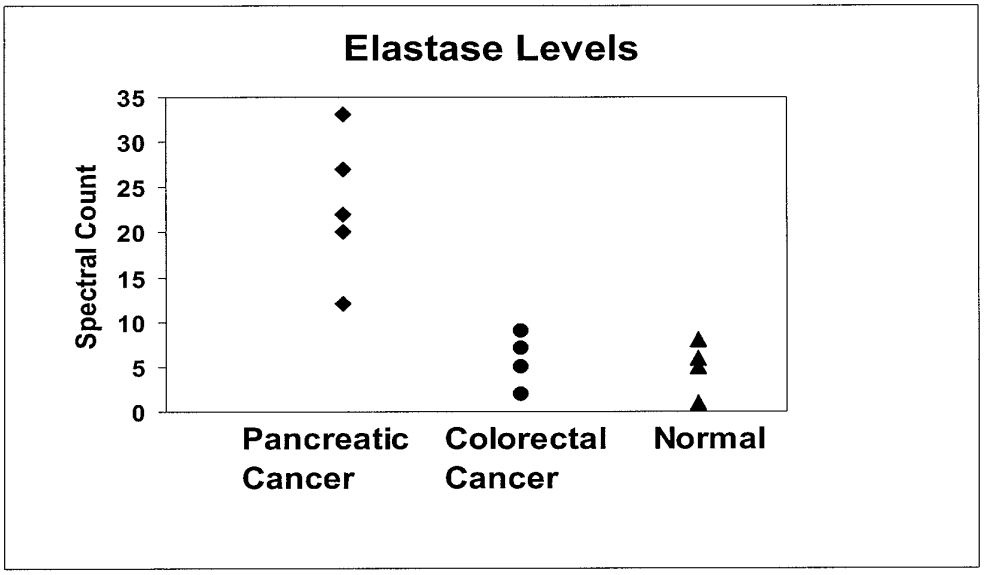

Elastase Levels
Spectral Count
35
30
25
20
15
10
5
0
Pancreatic Cancer
Colorectal Cancer
Normal

FIGURE 4

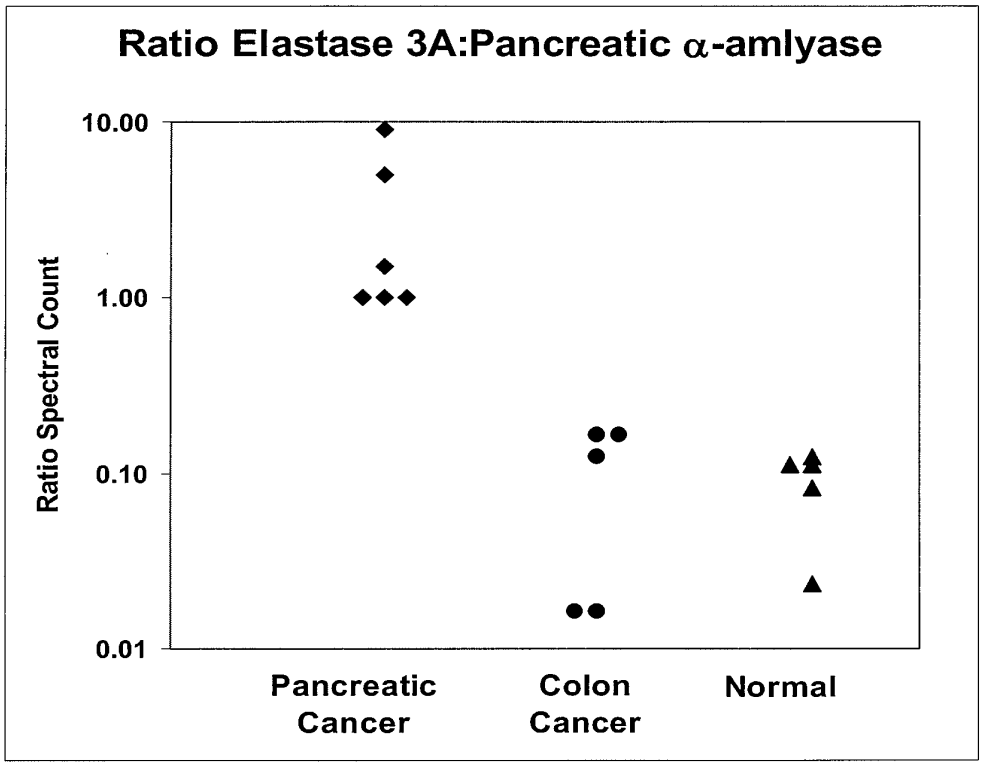

Ratio Elastase 3A:Pancreatic α-amlyase
Ratio Spectral Count
10.00
1.00
0.10
0.01
Pancreatic Cancer
Colon Cancer
Normal

FIGURE 5

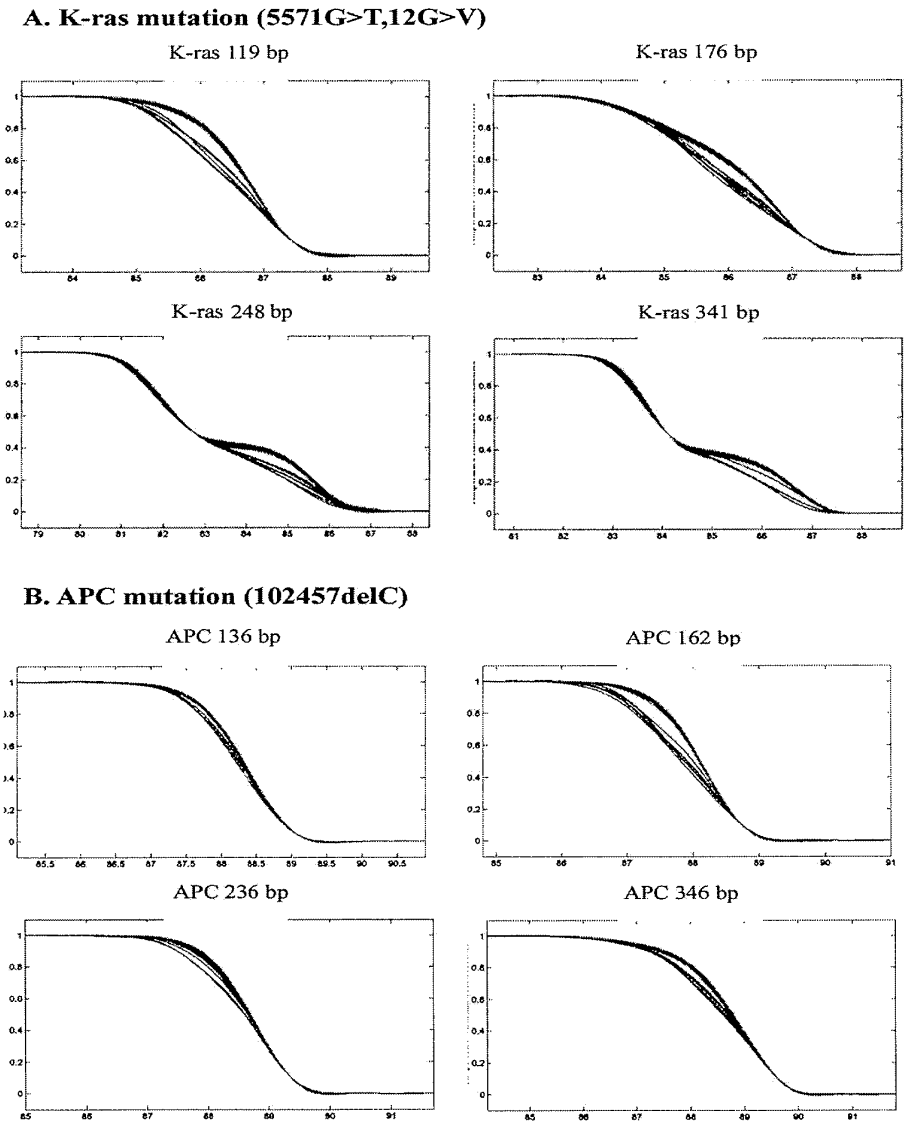

A. K-ras mutation (5571G>T,12G>V)
K-ras 119 bp
K-ras 176 bp
K-ras 248 bp
K-ras 341 bp
B. APC mutation (102457delC)
APC 136 bp
APC 162 bp
APC 236 bp
APC 346 bp

FIGURE 6

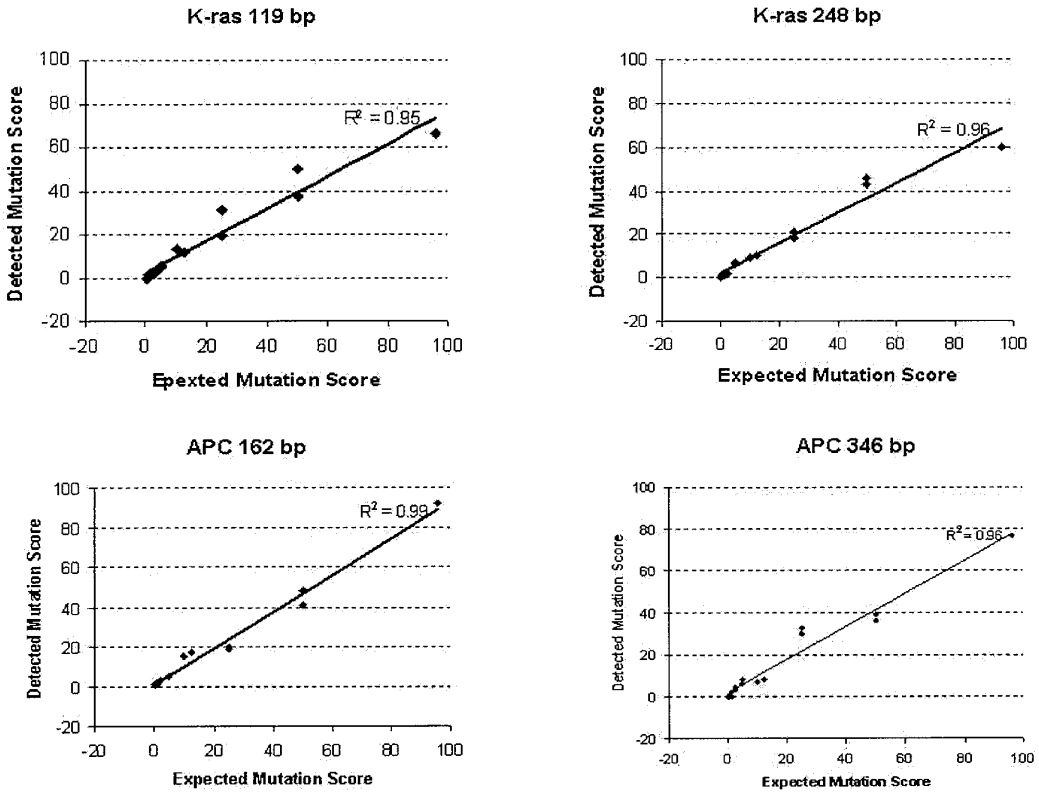

K-ras 119 bp
Detected Mutation Score
Epexted Mutation Score
R² = 0.95
K-ras 248 bp
Detected Mutation Score
Expected Mutation Score
R² = 0.96
APC 162 bp
Detected Mutation Score
Expected Mutation Score
R² = 0.99
APC 346 bp
Detected Mutation Score
Expected Mutation Score
R² = 0.96

FIGURE 7

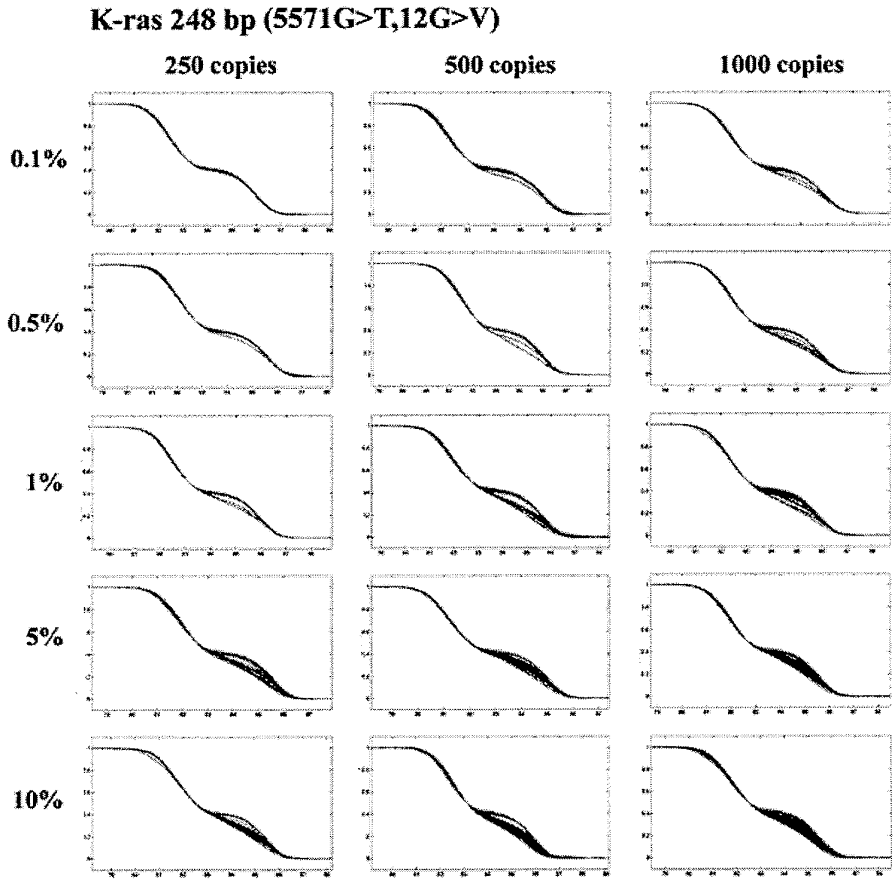

K-ras 248 bp (5571G>T,12G>V)
250 copies
500 copies
1000 copies
0.1%
0.5%
1%
5%
10%

FIGURE 8

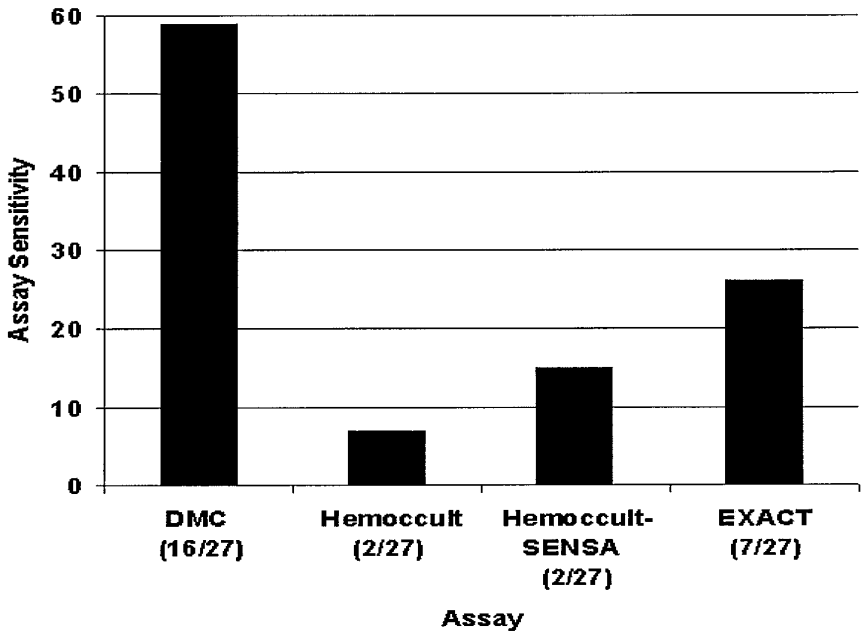
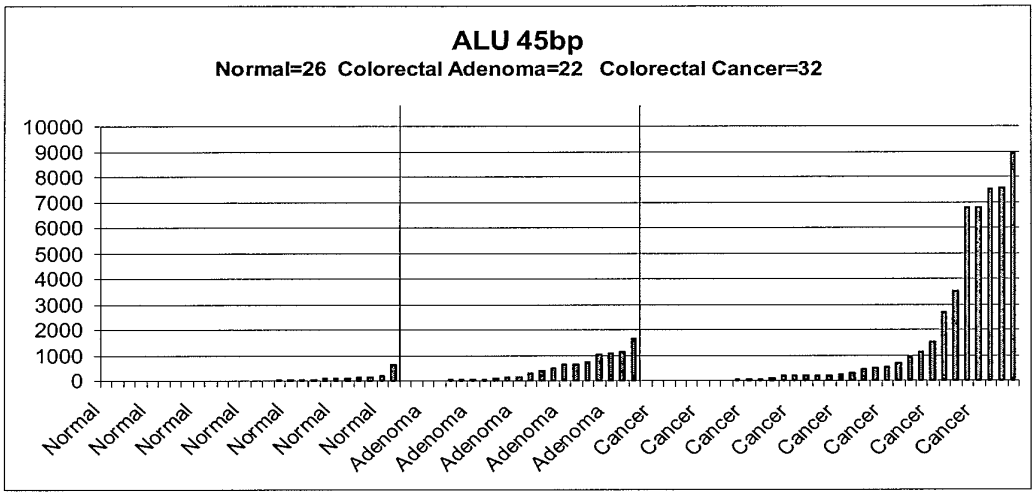

60
50
40
30
20
10
0
Assay Sensitivity
DMC (16/27)
Hemoccult (2/27)
Hemoccult-SENSA (2/27)
EXACT (7/27)
Assay

FIGURE 9


ALU 45bp
Normal=26 Colorectal Adenoma=22 Colorectal Cancer=32
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Normal
Adenoma
Cancer

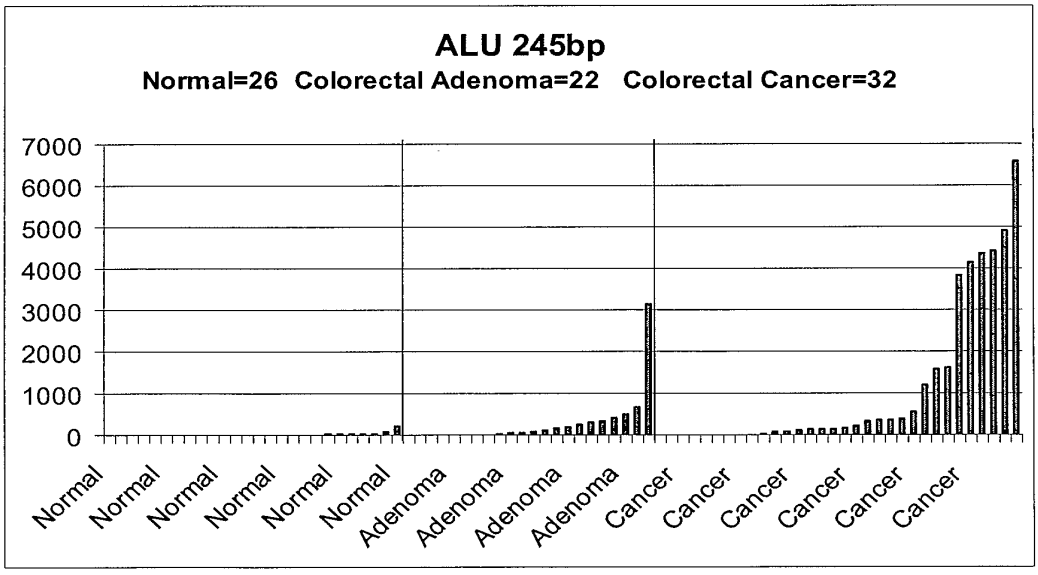
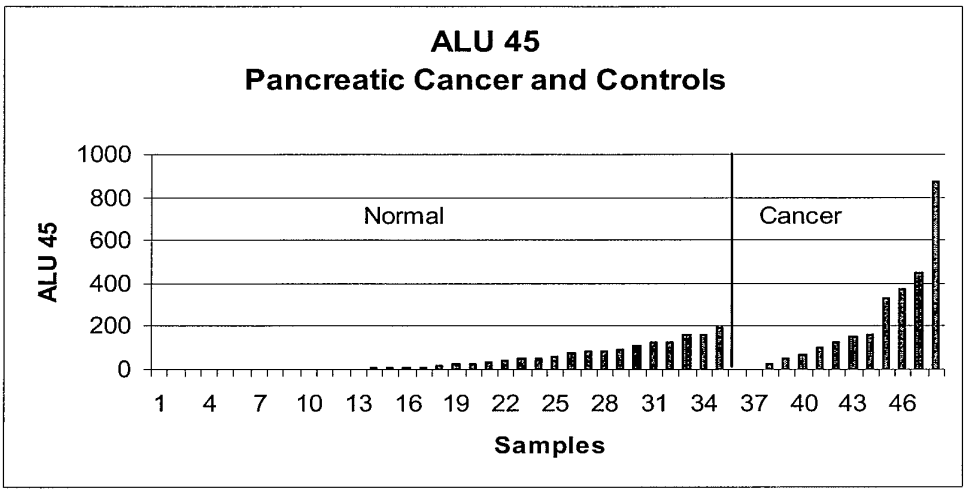

ALU 245bp
Normal=26 Colorectal Adenoma=22 Colorectal Cancer=32
7000
6000
5000
4000
3000
2000
1000
0
Normal
Adenoma
Cancer

FIGURE 10


ALU 45
Pancreatic Cancer and Controls
ALU 45
1000
800
600
400
200
0
Normal
Cancer
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46
Samples

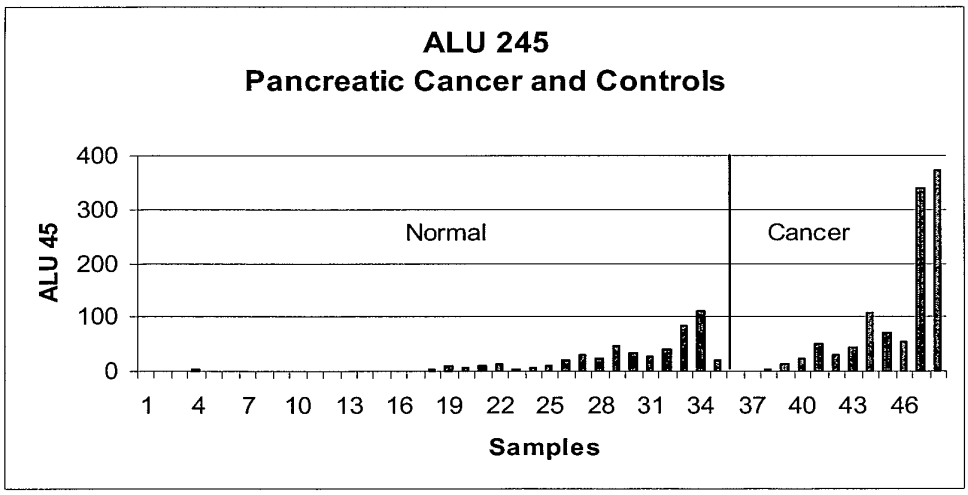

ALU 245
Pancreatic Cancer and Controls
ALU 45
400
300
200
100
0
Normal
Cancer
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46
Samples

FIGURE 11

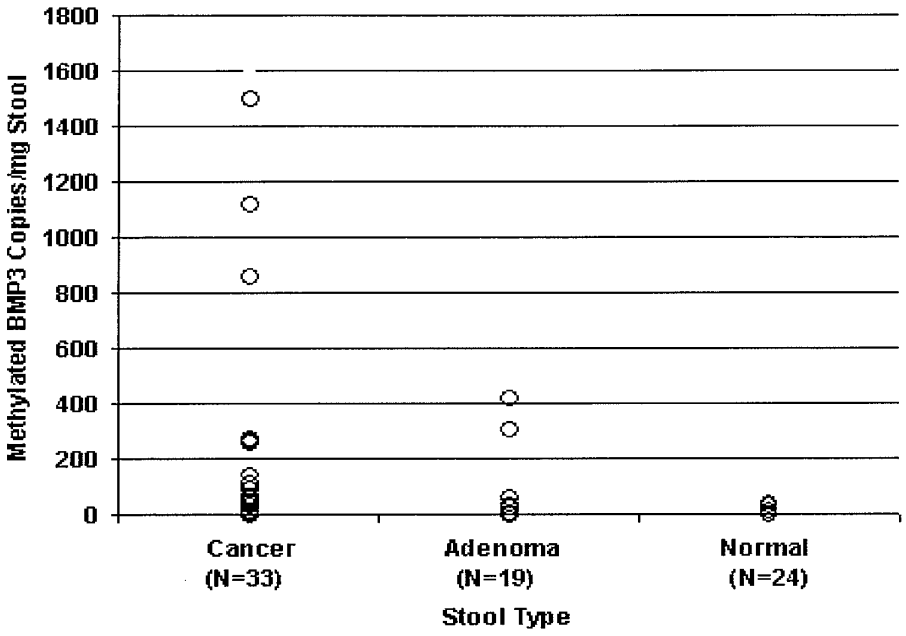
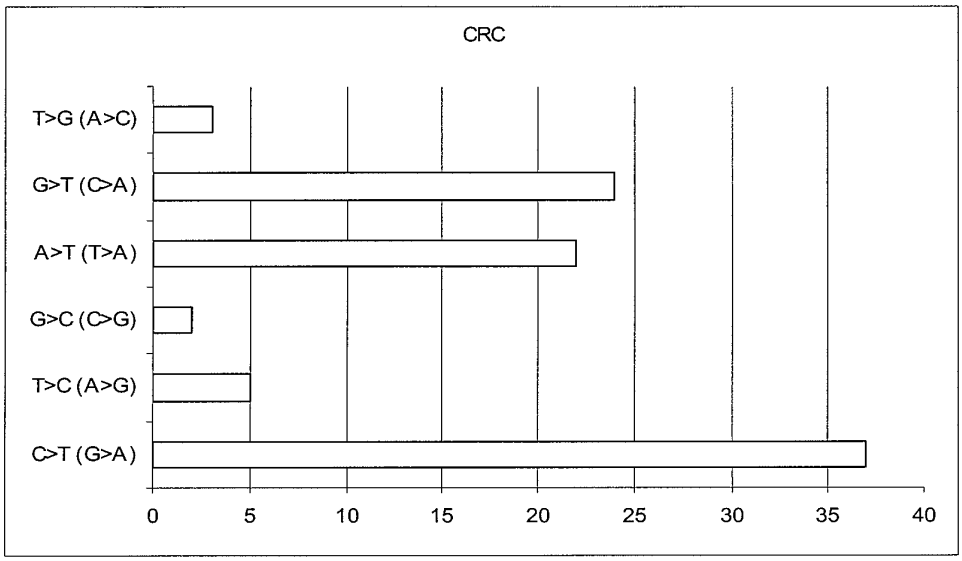

1800
1600
1400
1200
1000
800
600
400
200
0
Methylated BMP3 Copies/mg Stool
Cancer
(N=33)
Adenoma
(N=19)
Normal
(N=24)
Stool Type

FIGURE 12



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(54) **Title:** METHOD FOR STABILIZING BIOLOGICAL SAMPLES FOR NUCLEIC ACID ANALYSIS

(57) **Abstract:** Disclosed is a method for preparing nucleic acid containing biological samples for a nucleic acid integrity assay and/or multiple mutation analysis by incubating the biological sample with a stabilization solution that includes a buffer, a chelating agent and a salt.

WO 2005/113769 A1

METHOD FOR STABILIZING BIOLOGICAL SAMPLES FOR NUCLEIC ACID ANALYSIS

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Serial No. 60/571,120, entitled "Method for Stabilizing a Biological Sample for a Nucleic Acid Integrity Assay" filed on May 14, 2004, the disclosure of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention relates generally to assays to detect nucleic acid markers indicative of cancer and other diseases and more particularly to preparing nucleic acid-containing biological samples for use in these assays.

BACKGROUND OF THE INVENTION

Tissue and body fluid samples, including stools, contain shed cellular debris. In healthy patients, such debris is the result of apoptotic degradation as part of the normal cell cycle. Apoptosis reduces the integrity (intactness) of nucleic acids, proteins, and other cellular components in healthy individuals, so that only small fragments exist in the debris that results from the apoptotic process (e.g., exfoliated cellular debris).

In diseases in which cell cycle mechanisms are destroyed or impaired, cellular debris can include high-integrity cellular components, such as nucleic acids that have not been degraded by apoptosis. One class of disease in which cell cycle mechanisms are disrupted is cancer. An increased presence of high molecular weight nucleic acids in a biological sample therefore can reveal the presence of cancer in a patient from whom the biological sample was obtained. Disease detection assays known as nucleic acid integrity analysis assays have been developed that are based on the increased levels of non-degraded nucleic acid in a cancerous tissue or body fluid as compared to the level of non-degraded nucleic acid in a non-cancerous tissue or body fluid.

Nucleic acids in patient samples tend to degrade after they have been removed from the patient. This degradation can diminish the effectiveness of a nucleic acid integrity assay that scores a sample as diseased (e.g., cancerous) based on the presence of intact nucleic acids; if

the sample is excessively degraded, a sample that is actually positive may appear to be negative.

SUMMARY OF THE INVENTION

The invention is based in part on the discovery of a method for stabilizing nucleic acids in tissue and body fluid samples so as to facilitate analysis of the samples in a nucleic acid integrity assay. It has been unexpectedly found that contacting a patient sample with a stabilization solution stabilizes the DNA so that intact nucleic acids indicative of diseased cells are more effectively detected in a nucleic acid integrity assay.

Accordingly, in one aspect, the invention provides a method for preparing a nucleic acid sample for a nucleic acid integrity analysis assay. In one aspect, the invention provides a method for preparing a nucleic acid sample for a nucleic acid integrity analysis assay. The method includes providing a patient sample that includes shed cells or cellular debris and a nucleic acid and contacting the patient sample with a stabilization solution under conditions sufficient to stabilize the nucleic acid for nucleic acid integrity analysis. In one embodiment, the stabilization solution includes a buffer, a salt, and a chelating agent.

In some embodiments, the conditions are sufficient to detect at least a three-fold genomic equivalent (GE) increase in a nucleic acid integrity analysis of a patient sample having adenoma or cancer as compared to the GE detected in a nucleic acid integrity analysis of a sample from the patient that is not incubated with the stabilization solution. In one embodiment, the integrity analysis is performed by determining an amount of nucleic acid greater than about 200 bp in length using an assay that detects a nucleic acid (which can be a wild-type or mutant nucleic acid). The nucleic acid is present in a patient sample that includes shed cells or cellular debris. A patient is identified as having cancer or adenoma if the amount of nucleic acid is greater than an amount of nucleic acid expected to be present in a sample obtained from a patient who does not have cancer or adenoma.

The patient sample can be obtained from a patient that is, e.g., a vertebrate, including a mammal, a reptile, or an amphibian. A mammal can be, e.g., a human, a non-human primate (such as a gorilla or monkey, including a chimpanzee), a rodent (such as a mouse, rat, guinea pig, or gerbil) dog, cat, horse, pig, goat, sheep, or cow. The patient sample can be any body tissue or fluid that is suspected of containing DNA from a diseased cell (such as a precancerous or cancerous cell). In one embodiment, the patient sample is a stool sample.

In some embodiments, the patient sample can be obtained as part of a screen for, e.g., a disease or disease-associated condition that impairs, or could lead to impairment of, the proper function of the gastrointestinal system. Gastrointestinal diseases can include, e.g., those associated with the stomach, small intestine, and/or colon. The disease or condition can include cancers or precancerous conditions such as an adenoma. Other conditions include inflammatory bowel syndrome, inflammatory bowel disease, Crohn's disease, and others in which a genomic instability is thought to play a role.

In some embodiments, the patient sample is frozen and thawed prior to incubation with stabilization solution. In other embodiments, the patient sample is not frozen prior to incubation with the stabilization solution.

In general, the stabilization solution is added to the patient sample at a ratio of about 1 ml/gram of patient sample to about 20 ml/gram of patient sample. In some embodiments, the stabilization solution is provided at 1-15 ml/gram, 2-12 ml/gram, 3-11 ml/gram, or 4-7 ml/gram. However, higher or lower ratios may be used. When the patient sample is a stool sample, and the stabilization solution is 10 mM Tris-Cl, 1 mM EDTA, and 150 mM NaCL, a suitable ratio of stabilization solution to patient sample is 7 ml/gm.

In some embodiments, the patient sample and stabilization solution are incubated at about 4 to 28 degrees Centigrade. In some embodiments the temperature is 17 to 27 degrees Centigrade, e.g., about 20 to 25 degrees Centigrade. However, the sample and stabilization solution may be exposed to higher or lower temperatures (e.g., the sample and stabilization solution may be frozen). Also, a sample and buffer may be exposed to changing temperatures during transport and/or storage.

In various embodiments, the patient sample and stabilization solution are incubated at least 6 hours, e.g., at least 12 hours, at least 24 hours, or at least 36 hours.

In some embodiments, the buffer in the stabilization solution is 0.5 mM to 25 mM Tris, e.g., 5 mM to 15 mM Tris, 8 mM to 13 mM Tris or about 10 mM Tris. However, other buffers and/or concentrations may be used.

In some embodiments, the chelating agent in the stabilization solution is 0.01 to 2.5 mM EDTA, e.g., 0.75-1.25 mM EDTA, or 1mM EDTA. However, other chelating agents and/or concentrations may be used.

In some embodiments, the salt in the stabilization solution is 75 mM to 225 mM NaCl, e.g., 100 mM to 175 mM NaCl, or 150 mM NaCl. However, other salts (e.g., KCl etc.) and/or concentrations may be used.

In some embodiments, the stabilization solution is provided at pH 7.0 to 9.0, e.g., pH 7.5 to 8.5, or pH 8.0. However, higher or lower pHs may be used.

In some embodiments, the method further includes determining in the incubated patient sample an amount of nucleic acid greater than about 200 bp in length using an assay that detects wild-type or mutant nucleic acid, and identifying the patient as having cancer or adenoma if the amount is greater than an amount of nucleic acid expected to be present in a sample obtained from a patient who does not have cancer or adenoma (e.g., more than about 1.5 fold, 2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 50 fold, or more greater than an amount expected in a normal individual). In one aspect, a DNA integrity assay may include interrogating a sample for the presence of long DNA fragments (e.g., longer than 200 nucleotides, longer than 500 nucleotides, longer than 1,000 nucleotides, etc.) at two or more different loci. In one embodiment, a patient is identified as having cancer or adenoma if two or more loci (e.g., 3, 4, or more loci) are positive for the presence of abnormally high levels of long DNA.

In one embodiment, the invention provides a method for preparing a nucleic acid sample for a nucleic acid integrity and/or multiple mutation analysis assays for diagnosing a carcinoma or adenoma. The method may include providing a patient stool sample that includes shed cells or cellular debris and a nucleic acid, and incubating the patient sample with a stabilization solution under conditions sufficient to stabilize the nucleic acid for nucleic acid integrity and/or multiple mutation analysis. The stabilization solution may be about pH 7.5 to about pH 8.5 and may include 0.5 mM to 25 mM Tris, 0.01 to 2.5 mM EDTA and 100 mM to 200 mM NaCl. For example, the stabilization solution can be 10 mM Tris-Cl pH 8.0, 1 mM EDTA, and 150mM NaCl. In one embodiment, the conditions are sufficient to detect at least a three-fold genomic equivalent (GE) increase in a nucleic acid integrity analysis of a patient sample having adenoma or cancer as compared to the GE detected in a nucleic acid integrity analysis of a sample from the patient that is not incubated with the stabilization solution.

Aspects of the invention may be used for transporting or storing biological samples after they are obtained and before they are processed for analysis. For example, methods of the invention may be used to stabilize nucleic acid in biological samples (e.g., stool samples) for about 12 hours, about 24 hours, about 36 hours, or longer (e.g., 4 days, 5, days, 6 days, 1 week, or longer), even in the absence of refrigeration or freezing.

Aspects of the invention may be particularly useful for detecting indicia of adenomas, early stage cancers, and/or other diseases that may be characterized by very low frequencies of mutant nucleic acid in a sample.

Aspects of the invention may be particularly useful for preserving biological samples for nucleic acid integrity assays or for multi-panel screens that include one or more nucleic acid integrity assays. Aspects of the invention may be particularly useful for detecting mutations in samples that contain very little human DNA (e.g., DIA negative samples). According to the invention, the amount of human DNA recovered from stool from different subjects may vary and it may be particularly important to preserve nucleic acids in samples that contain low amounts of human DNA.

Accordingly, aspects of the invention may be useful for screening a population to identify individuals with indicia of disease, and for avoiding or reducing the number of false negatives (subjects with a disease but who are identified as normal or healthy) in such screens. In one embodiment, average-risk individuals may screened for one or more indicia of a sporadic disease (e.g., adenoma, cancer, etc.). In one embodiment, high-risk individuals may be screened for a sporadic disease or a disease that may be inherited. In one embodiment, a screen may be performed on a population of individuals regardless of their risk profile.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present Specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Table (Table 2) showing the results of DNA Integrity Assay (DIA) analysis performed on stool samples under different experimental conditions.

Figure 2 is a Table (Table 5) showing the results of DNA Integrity Assay (DIA) analysis performed on stool samples under different experimental conditions.

DETAILED DESCRIPTION OF THE INVENTION

It has been unexpectedly found that a DNA integrity signal can be stabilized and/or enhanced by mixing, or incubating, a patient sample known to or suspect of containing DNA indicative of a disease with a stabilization solution prior to performing the DNA integrity assay. The stabilization solution typically includes one or more buffers, and/or chelating agents, and/or salts. Aspects of the invention are particularly useful for nucleic acid integrity assays. However, mutation detection (for example, in a multiple mutation assay) and hypermethylation analysis also can benefit from aspects of the invention.

According to the invention, an important challenge for colon cancer detection from stool is to preserve the integrity of human DNA in the hostile stool environment, in order to recover, amplify, and interrogate the DNA for known cancer related abnormalities. Nucleases that are active in stool have the potential to rapidly degrade DNA, including the minor human DNA component, and measures may be taken to minimize their negative impact. Typically, clinical samples are frozen as quickly as possible after collection. However, in order to use fecal DNA tests in population screens, it should be expected that there will be some variability in the time between sample collection and shipping to testing labs, and furthermore, some variability in the temperature at which stool samples are transported. In order to eliminate any variables in sample handling that might have an impact on assay performance we have run controlled sample incubation experiments and looked at how different markers in a multi-target assay are affected.

Markers may be chosen that yield an acceptable clinical sensitivity for the intended application such as screening a population for indicia of a disease. In addition, for stool sample analysis, mutation detection methods should offer sufficient analytical sensitivity since the human DNA recovered from stool is highly heterogeneous. Normal cells are sloughed into the colonic lumen along with the mutant cells. Therefore, in one embodiment, analytical methods should detect as little as 1% (or less) mutant DNA in the presence of excess wild-type DNA. Also, certain sample preparation methodologies may be used for maximum recovery of human DNA from samples. The vast majority of DNA recovered from stool often is bacterial in origin, with the human DNA component representing only a small minority. Certain purification methodologies can efficiently select for the rare human component, and since the mutant copies (when they exist) represent only a small percentage of the total human DNA from stool it may be important to maximize the recovery of human DNA in order to maximize the probability of amplifying mutant copies in the PCR reactions. In one embodiment, gel

electrophoresis methods for capturing human DNA may be used. 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. However, in order to provide the option of decentralized sample analysis and still retain maximum sample integrity, it is desirable to use a more robust and standardized sample handling method.

In one aspect, the invention provides methods for stabilizing biological samples (e.g., stool samples) by adding a stabilization solution to a sample as soon as possible after the sample is obtained. Methods of the invention do not require refrigeration or freezing. Aspects of the invention are based, in part, on the surprising discovery that nucleic acids in certain biological samples are stable at room temperature for hours, and even days (e.g., 1 day, 2 days, 3 days, or longer). However, in certain embodiments, samples with stabilization solution may be refrigerated or frozen. Aspects of the invention are particularly useful for preserving samples for nucleic acid integrity analysis. However, methods of the invention may be used to preserve samples for other assays including mutation detection and/or hypermethylation assays. In certain embodiments, methods of the invention are used to preserve a sample for analysis using a nucleic acid integrity assay along with a mutation detection assay (e.g., a multiple mutation panel assay), a hypermethylation assay, or both.

Nucleic acid integrity assays are known in the art and are described in, e.g., US Patent Application No. 20040043467, US Patent Application No. 20040014104, US Patent No. 6,143,529, and Boynton et al., Clin. Chem. 49:1058-65, 2003. Nucleic acid integrity assays are based on higher levels of intact nucleic acid that appear in debris from cells that lyse non-apoptoTically. Healthy patient generally produces cellular debris through normal apoptotic degradation, resulting in relatively small fragments of cellular components in tissue and body fluid samples, especially luminal samples. Patients having a disease generally produce cells and cellular debris, a proportion of which has avoided normal cell cycle regulation, resulting in relatively large cellular components. As a result, the disease status of a patient is determined by analysis of patient cellular components produced in specimens obtained from the patient. The presence of such fragments is a general diagnostic screen for disease.

Nucleic acids in patient samples tend to degrade after they have been removed from the patient. This degradation can diminish the effectiveness of a nucleic acid integrity assay that scores a sample as diseased (e.g., cancerous) based on the presence of intact nucleic acids; if the sample is excessively degraded, a sample that is actually positive may appear to be negative. While not wishing to be bound by theory, it is postulated that the stabilization buffer of the invention inhibits the nucleases that degrade the nucleic acids present in the diseased patient samples.

In some aspects of the invention, the addition of a stabilization solution to a biological sample may be used to preserve nucleic acid molecules containing one or more mutations that may be detected in a multiple mutation analysis (e.g., an analysis that involves interrogating a sample for the presence of a mutation at one or more loci, for example at about 2, about 3, about 4, about 5, about 10, about 15, about 20, about 25, or more loci). In some embodiments, the addition of a stabilization solution may be used to preserve nucleic acid for a methylation specific analysis to detect the presence of hyper-methylated nucleic acid molecules at one or more loci that may be indicative of cancer, adenoma, or other disease. In some embodiments, the addition of a stabilization solution may be used to preserve nucleic acid for a combination of a nucleic acid integrity assay and/or a multiple mutation analysis and/or a methylation detection assay. Assays may be performed under conditions to detect a small amount of mutant nucleic acid in a heterogeneous sample containing an excess of non-mutant nucleic acid (e.g., where the mutant nucleic acid represents less than 10%, less than 5%, less than 1%, or about 0.1% or less of the nucleic acid at a particular locus). In some embodiments, a digital assay may be performed on the preserved nucleic acid in order to detect rare genetic events. In some aspects, stabilization methods of the invention may preserve more than 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of the nucleic acids indicative of a disease (e.g., long nucleic acid fragments, nucleic acid molecules containing one or more specific mutations, and/or hyper-methylated nucleic acid molecules).

In general any body organ, tissue, or fluid known to or suspected of containing nucleic acid that can be characterized in a nucleic acid integrity analysis, multiple mutation assay, or methylation study may be used. Suitable patient samples include those likely to contain sloughed cellular debris. Such specimens include, but are not limited to, stool, blood serum or plasma, sputum, pus, colostrum, and others. In diseases, such as cancer, in which genomic instabilities or abnormalities have interfered with normal cell cycle regulation, specimens such as those identified above contain relatively intact fragments of cellular components.

The stabilization solution can be applied to a biological sample that is isolated directly from a patient, i.e., a freshly isolated biological sample. Alternatively, the method can be used on a biological sample that has been frozen (e.g., at -20 °C or -80 °C).

In aspects of the invention, stabilization solution may be added to a biological sample at any suitable ratio of sample to buffer. Ratios may be determined as a weight to volume (w/v) ratio. In some embodiments, the ratio may be about 1:1, about 1:2, about 1:3, about 1:4, about 1:5, about 1:6, about 1:7, about 1:8, about 1:9 or about 1:10 (w/v) sample to stabilization solution. However, higher or lower ratios may be used.

In aspects of the invention, a biological sample may be weighed before a buffer is added. In some embodiments, a sample (e.g., a stool sample) may weigh about 5 g, about 10 g, about 15 g, about 20 g, about 25 g, about 30 g, about 35 g, about 40 g, about 45 g, about 50 g, about 55 g, about 60 g, about 65 g, about 70 g, about 75 g, about 80 g, about 85 g, about 90 g, about 95 g, about 100 g, or more. It should be appreciated that a large amount of sample (e.g., over 25 g, 30 g, 50 g, 100 g, or more of stool) may be required in order to detect conditions such as adenomas or early stage cancers where a very small amount of mutant or abnormal nucleic acid may be present in a sample. According to the invention, it may be particularly important to immediately stabilize biological samples with a stabilization solution when the samples are to be interrogated for indicia of adenoma or early stage cancer. However, it also may be useful to stabilize biological samples for detecting indicia of later stage diseases.

In general, a stabilization solution may include one or more buffers and/or one or more chelating agents and/or one or more salts, or any combination of two or more thereof. The choices of buffer, chelating agent, and salt can be determined by the artisan. The suitability of a particular stabilization solution can be determined by comparing a nucleic acid integrity assay on samples that have been incubated with the stabilization solution to a parallel biological sample that has not been incubated with the stabilization solution. A suitable stabilization solution is a solution that shows a significant average fold increase in genome equivalents (GE) in a nucleic acid integrity assay compared to a GE determination made on parallel samples that have not been treated with the stabilization solution. Methods of calculating genome equivalents (GE) are known in the art (see, e.g., e.g., US Patent Application No. 20040043467, US Patent Application No. 20040014104, US Patent No. 6,143,529, and Boynton et al., Clin. Chem. 49:1058-65, 2003) and are illustrated in the Examples.

The temperature and pH optimum can also be determined empirically and optimized according to the combination of buffer, chelating agent and salt in the stabilization solution. While room temperature has been found to be a suitable temperature for incubating the patient sample and the stabilization solution, higher or lower temperatures (e.g., 4 °C to 16 °C or 25 °C to about 37 °C) can also be used, provided they do not undermine the effectiveness of the stabilization solution. The mixed patient sample and stabilization solution is preferably subjected to a minimum of agitation. However, according to the invention, the addition of a stabilization solution with little or no agitation is surprisingly effective at preserving nucleic acids for subsequent analysis.

In one aspect of the invention, a stabilization solution may be particularly useful when samples are not refrigerated or frozen or when there is a risk that a sample may not be maintained at a sufficiently low temperature to preserve indicia of disease. For example, a stabilization solution may be particularly useful if a sample is obtained at a remote location and mailed or delivered to a testing center. However, stabilization solutions also may be useful to preserve samples that are being processed on-site at a medical center.

Buffers

Suitable buffers include, e.g. tris(hydroxymethyl)aminomethane, sodium phosphate, sodium acetate, MOPS, and other buffering agents as long as a buffer has the capacity to resist a 0.1 to 1 molar tris(hydroxymethyl)aminomethane or 0.1 to 1 molar phosphate ion. A combination of buffering agents can be used, so long as the solution has the required buffering capacity. Methods for determining the buffering capacity of a solution are well known in the art.

The comparison of buffering capacity is preferably carried out in the presence of the salt and chelating agent to be used in the stabilization solution, at the salt concentration to be used, and with the solutions being compared at about the same temperature, preferably at a temperature within the range of about 15° C to about 25° C.

Chelating Agents

Table 1 provides a representative list of chelating agents that can be used in the stabilization solution. The list provided in Table 1 is not meant to be exhaustive. In some embodiments, chelating agents are those which bind trace metal ions with a binding constant ranging from 101 to 10100; in some embodiments, chelating agents bind trace metal ions with

a binding constant ranging from 1010 to 1080 ; in some embodiments, the chelators bind trace metal ions with a binding constant ranging from 1015 to 1060.

Table 1. Examples of Chelating Agents

CHELATORS	
ABBREVIATION	FULL NAME
EDTA free acid	Ethylenediamine-N,N,N',N',-tetraacetic acid
EDTA 2Na	Ethylenediamine-N,N,N',N',-tetraacetic acid, disodium salt, dihydrate
EDTA 3Na	Ethylenediamine-N,N,N',N',-tetraacetic acid, trisodium salt, trihydrate
EDTA 4Na	Ethylenediamine-N,N,N',N'-tetraacetic acid, Tetrasodium salt, tetrahydrate
EDTA 2K	Ethylenefismin-N,N,N',N'-tetraacetic acid, dipotassium salt, dihydrate Ethylenefisminr-N,N,N',N'-tetraacetic acid, dipotassium salt, dihydrate
EDTA 2Li	Ethylenediamine-N,N,N',N'-tetraacetic acid, dilithium salt, monhydrate
EDTA $2NH_4$	Ethylenediamine-N,N,N',N'-tetraacetic acid, diammonium salt
EDTA 3K	Ethylenediamine-N,N,N',N'-tetraacetic acid, Tripotassium salt, dihydrate
Ba(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, barium chelate
Ca(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, calcium chelate
Ce(III)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, cerium chelate
Co(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, cobalt chelate
Cu(11)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, copper chelate
Dy(III)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, dysprosium chelate
Eu(III)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, europium chelate
Fe(III)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, iron chelate
In(III)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, indium chelate
La(111)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, lanthanum chelate
Mg(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, magnesium chelate
Mn([1)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, manganese chelate
Ni(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, nickel chelate
Sm(III)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, samarium chelate
Sr(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, strontium chelate
Zn(II)-EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid, zinc chelate
CyDTA	trans-1 ,2-Diaminocyclohexane-N,N,N',N'-tetraaceticacid,monohydrate

DHEG	N,N-Bis(2-hydroxyethyl)glycine
DTPA-OH	1,3-Diamino-2-hydroxypropane-N,N,N',N'-tetraacetic acid
DTPA	1,3-Di aminopropane-N,N,N',N'-tetraacetic acid
EDDA	Ethylenediamine-N,N'-diacetic acid
EDDP	Ethylenediamine-N,N'-dipropionic acid dihydrochioride
EDDPO	Ethylenediamine-N,N'-bis(methylenephosphonic Acid), hemihydrate
EDTA-OH	N-(2-Hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid
EDTPO	Ethylenediamine-N,N,N',N'-tetrakis(methylenephosponic acid)
EGTA	O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid
HBED	N,N-bis(2-hydroxybenzyl)ethylenediamine- N,N-diacetic acid
HDTA	1,6-H ex am ethyl enediamine-N,N,N',N'- tetraacetic acid
HIDA	N-(2-Hydroxyethyl)irninodiacetic acid
IDA	Iminodiacetic acid
Methyl- EDTA	1,2-Diaminopropane-N,N,N',N'-tetraacetic acid
NTA	Nitrilotriacetic acid
NTP	Nitrilotripropionic acid
NTPO	Nitrilotris(methylenephosphoric acid), trisodium salt
O-Bistren	7,19,30-Trioxa-1,4,10,13,16,22,27,33 -octaabicyclo [11,11,11] pentatriacontane,
TTHA	Triethylenetetramine-N,N,N',N",N"',N"'- hexaacetic acid

Salts

Candidate salts include, e.g, NaI, NaBr, NaCl, LiCl, KCl, KI, KBr, CsCl, GNHCl and GNSCN. In some embodiments, the salt is chaotropic and has an anion such as perchlorate, iodide, thiocyanate, acetate, trichloroacetate, hexafluorosilicate, tetrafluoroborate and the like. Cations for a chaotropic salt can include, e.g., the elements lithium, sodium, potassium, cesium, rubidium, guanidine and the like. More than one salt can be present in the buffered aqueous salt solution.

Patient samples that have been treated with a stabilization solution can be subject to the nucleic acid integrity assay. For example the treated sample can be used in method that includes determining an amount of nucleic acid greater than about 200 bp in length using an assay that detects wild-type or mutant nucleic acid, wherein said nucleic acid is present in a patient sample comprising shed cells or cellular debris; and identifying the patient as having cancer or adenoma if said amount is greater than an amount of nucleic acid expected to be present in a sample obtained from a patient who does not have cancer or adenoma.

Adenomas

According to aspects of the invention, stabilization solutions may be particularly useful for preserving nucleic acids to detect one or more indicia of adenomas. Adenomas may be early indicia of cancer, for example colon cancer. Not all adenomas become cancers. However, many cancers (e.g., carcinomas such as colorectal carcinomas) are thought to develop from adenomas. Indeed, a majority of colon cancers are thought to develop from adenomas. Therefore, detecting adenomas is particularly useful for identifying early signs or risks of colorectal cancer (e.g., cancerous and precancerous lesions or growths in the colon).

Adenomas may be invasive adenocarcinomas, significant adenomas, and low potential polyps. Invasive adenocarcinomas may be, for example, adenocarcinomas at different TNM stages (e.g., TNM stages 1, 2, 3, or 4). Significant adenomas may be, for example, carcinomas in-situ/high-grade dysplasias (CIS/HGD) having a diameter of greater than 1 cm, about 1 cm, less than 1 cm, or of unknown size; vilous adenomas having a diameter of greater than 1 cm, about 1 cm, less than 1 cm, or of unknown size; tubulovillous adenomas having a diameter of greater than 1 cm, about 1 cm, less than 1 cm, or of unknown size, and low-grade dysplasias (LGD) with a diameter of greater than or equal to 1 cm. Low potential polyps may be, for example advanced polyps, and adenoma low-grade dysplasias (LGD) with an unknown diameter or a diameter of less than 1 cm.

According to aspects of the invention, adenomas can be detected at different positions in the colon and rectum (including the right and left colon and the transverse colon).

In one embodiment, the following panel may be used to detect adenomas with greater than 60% sensitivity: assays may be performed to detect one or more genetic abnormalities from a multiple mutation panel of genetic abnormalities at 22 loci including KRas mutations in codon 12 (K12p.1, K12p.2) and codon 13 (K13p.2); mutations in APC codons 1309 (deletions), 1306 (mutations at position 1), 1312 (mutations at position 1), 1367 (mutations at position 1), 1378 (mutations at position 1), 1379 (mutations at position 1), 1450 (mutations at position 1), 1465 (deletions), 876 (mutations at position 1) and 1554 (insertions); mutations in P53 codons 175p.2, 245p.1, 245p.2, 248p.1, 248p.2, 273p.1, 273p.2 and 282p.1; and deletions at the BAT-26 locus. Mutations at these loci can be detected using primer extension assays (including single base extension assays and assays designed to detect micro-satellite instability such as BAT-26 deletions) or other assays that are useful to detect one or more of these genetic abnormalities.

In another embodiment, the following panel may be used to detect adenomas with greater than 60% sensitivity: assays are performed to detect hypermethylation at one or both of the HLTF and V29 loci. Hypermethylation at these loci can be detected using methylation specific primer analysis (e.g., MSP amplification) or other assays that are useful to detect hypermethylation at one or more of these genetic loci.

In one embodiment, scanning for one or more mutations at the APC-MCR may detect adenomas with greater than 74% sensitivity.

In one embodiment, the following panel may be used to detect adenomas with greater than 90% sensitivity: scanning for one or more mutations in the APC-MCR locus, exon 9 of the PIK3CA locus, exon 20 of the PIK3CA locus, B-catenin (e.g., exon 5), or a mutation in BRAF that results in a V599E amino acid change. Scanning as described herein can be used to detect one or more mutations in the APC-MCR locus, exon 9 of the PIK3CA locus, or exon 20 of the PIK3CA locus. Mutations at the BRAF locus can be detected via primer extension or other appropriate methodology.

In one embodiment, a combination of all of the above loci may be used to detect adenomas with a greater than 95% sensitivity (e.g., greater than 98% sensitivity).

Any of the assays described herein may be performed in a digital format wherein a sample is diluted into aliquots wherein each aliquot contains on average between 1 and 20 target molecules of DNA for analysis (e.g., between 1 and 10 molecules, between 1 and 5 molecules, etc.).

The invention will be further illustrated in the following non-limiting examples.

EXAMPLES

Example 1: Materials and Methods

Sample collection and incubation

Samples were collected from known CRC patients as well as patients without cancer by a separate organization (Indivumed GmbH; Hamburg, Germany) that also managed all patient informed consent and compliance with human subject guidelines. All stool samples were frozen within 1 hour of defecation, and shipped to EXACT Sciences on dry ice (-78°C). Once received, samples were subjected to prescribed room temperature incubation times as described below. Prior to the start of the incubation time-course stool samples were thawed and one aliquot was processed to recover DNA immediately (to). The DNA from the to-aliquot for all

samples was analyzed and served as an incubation control. The remainder of the stool was left to incubate at room temperature. At prescribed time points aliquots were removed from the stool, human DNA recovered and analyzed in similar manner to the controls.

All aliquots were standardized by weight (30 g). An experiment was designed to measure the effect of incubation time on DNA integrity, as well as the quantity of recoverable DNA. The experiments also included an addition of stabilization buffer to stool aliquots. Stabilization buffer consisted of 0.5M Tris, 0.15M EDTA, and 10mM NaCl (pH 9.0). In these experiments aliquots were stored at room temperature for 36 or 48 hours, with and without buffer added. In the case of buffer addition, the buffer was simply added to the stool aliquot, in a plastic container with a lid, but no effort was made to homogenize the sample. At the prescribed time period, the aliquots with and without buffer were processed to recover human DNA, and then analyzed by the DIA assay. An additional set of experiments was conducted to study the effect of incubation time on specific gene mutations. In this experiment one set of samples (6 samples) was incubated for 36, 48, or 72 hours without any stabilization buffer added. Another set of samples (5 samples) was incubated for 36 or 48 hours, with and without buffer added. After the prescribed incubation time all samples were processed to recover and purify human DNA, and analyze the DNA for gene mutations as described below.

Recovery of DNA from stool

The sample preparation methodology used to recover DNA from stool was previously reported (Ahlquist DA, Skoletsky JE, Boynton KA, et al. Colorectal cancer screening by detection of altered human DNA in stool: Feasibility of a multi-target assay panel. Gastroenterology 2000; 119:1219-1227; Whitney D, Skoletsky J, Moore K, et al. Enhanced Retrieval of DNA from Human Fecal Samples Results in Improved Performance of Colorectal Cancer Screening Assay. J Mol. Diagn. 2004; 6 (4), 386-395). Stool aliquots were weighed and combined with Exact buffer A (1:7 (w/v) ratio), and homogenized on an Exactor (Exact Sciences). After homogenization, a 4-g stool equivalent (-32 mls) of each sample was centrifuged to remove all particulate matter. The supernatants were then treated with 20ul TE buffer (0.01 mol/L Tris [pH 7.4] and 0.001 mol/L EDTA) containing RNase A (2.5 mg/mL), and incubated at 37°C for 1 hour. Total nucleic acid was then precipitated (first adding 1/10 volume 3 mol/L NaAc, then an equal-volume of isopropanol). Genomic DNA was pelleted by centrifugation, the supernatant removed, and the DNA resuspended in TE.

Human DNA Purification

Target human DNA fragments were purified from total nucleic acid preparations using a newly developed DNA affinity electrophoresis purification methodology. This method has recently been described in detail 9. In brief, human DNA can be separated from the excess bacterial DNA by hybridization of the target sequences to complementary, covalently-bound oligonucleotide capture probes in acrylamide gels membranes. Crude human DNA preparations (2400 ul) were mixed with 960 ul formamide (Sigma), 385 ul 10x TBE, and filtered through a 0.8 um syringe filter (Nalgene, Rochester, NY), then denatured (heated at 95C for 10 min, then cooled in ice for 5 min). The sample mix was loaded on top of the capture membrane, and electrodes above and below the capture layer were applied. Samples were electrophoresed (15V, 16h) using TBE in the reservoirs above and below the capture layer. After electrophoretic capture the remaining solution was removed from the tubes, and the tube array was separated from the capture plate. The capture membranes were then washed and 40ul of 100mM NaOH was added to the top of the capture membrane and incubated for 15 min. The capture plate placed on top of a custom molded 48-well DNA collection plate and centrifuged briefly (1900xg) to recover the eluted DNA. Then 8ul of neutralization buffer (500mM HCL + O.lx TE) was added to each well of the collection plate and mixed.

Quantification of recovered human DNA by TaqMan analysis

TaqMan analysis was performed on an I-Cycler (BioRad) with primers against a 200-bp region of the APC gene. A probe labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) was used to detect PCR product. Amplification reactions consisted of captured human stool DNA mixed with 10x PCR buffer, LATaq enzyme (Takara), lx PCR primers (5 μM), and lx TaqMan probe (2 μM; Biosearch Technologies). We used 5μl of captured DNA in the PCR reactions. TaqMan reactions were performed with the same program as described above (DIA).

Sequence-specific amplification

Polymerase chain reaction (PCR) amplifications (50 μL) were performed on MJ Research Tetrad Cyclers (Watertown, MA) using 10 μL of purified DNA, 10x PCR buffer (Takara Bio Inc; Madison, WI), 0.2 mmol/L dNTPs (Promega, Madison, WI), 0.5 μmol/L sequence-specific primers (Midland Certified Reagent Co., Midland, TX), and 2.5 U LATaq DNA polymerase (Takara). All amplification reactions were performed under identical

thermocycler conditions: 94°C for 5 minutes, and 40 cycles consisting of 94°C (1 min.), 60°C (1 min.), and 72°C (1 min.), with a final extension of 5 minutes at 72°C. Thirteen separate PCR reactions were run per sample. For analysis of each of the PCR products, 80, of each amplification reaction was loaded and electrophoresed on a 4% ethidium bromide-stained NuSieve 3:1 agarose gel (FMC, Rockland, ME) and visualized with a Stratagene EagleEye II (Stratagene, La Jolla, CA) still image system.

The multi-target assay was designed to have 13 separate PCR reactions in the multiple mutation (MuMu) panel, and 16 PCR reactions in the DIA portion of the assay.

Mutation panel analysis

The presence or absence of point mutations or Bat-26-associated deletions was determined by using modified solid-phase single-base extension (SBE) reactions. Point mutation targets included; codons K12p1, K12p2, and K13p2 on the K-ras gene; codons 876, 1306, 1309, 1312, 1367pl, 1378pl, 1379, 1450p1, 1465 and 1554 on the APC gene; and codons 175p2, 245pl, 245p2, 248pl, 248p2, 273pl, 2'73p2, and 282pl on the p53 gene. Including the Bat-26 deletion marker, the panel consisted of 22 markers in total. For all gene targets, separate wild-type and mutant specific reactions were performed. Details of the reactions and analysis using capillary electrophoresis have been previously described (Whitney D, Skoletsky J, Moore K, et al. Enhanced Retrieval of DNA from Human Fecal Samples Results in Improved Performance of Colorectal Cancer Screening Assay. J Mol. Diagn. 2004; 6 (4), 386-395).

DNA integrity assay (DIA)

The DIA assay has been previously described in detail. More recently this assay has been converted to a real-time PCR methodology. Three unique PCR reactions (in duplicate) per loci were run on I-Cycler instruments (BioRad; Hercules, CA). The strategy was to capture locus specific segments and perform small (-100bp) PCR amplifications remote from the capture site as an indicators of DNA length. DNA fragments for integrity analysis were amplified from four different loci: 17p13; 5q21; HRMT1L1; LOC91199 (named DIA-D, DIA-E, DIA-X, and DIA-Y, respectively). PCR primer sets and associated TaqMan probe for each locus of interest are "walked" down the chromosome thereby interrogating for the presence and quantitation of increasing length of DNA of approximately, 100bp, 1300 bp, 1800 bp and 2400 bp fragments of captured DNA. Purified DNA template (5µl) was mixed with, 5µl 10x PCR buffer (Takara), 10111 dNTP's (2mM) (Promega), 0.25111 LATaq (5 U/µl; Takara), 24.751.11

molecular biology grade water (Sigma), 5µl of a mix of PCR primers (5µM; Midland) and TaqMan dual-labeled probes (2µM; Biosearch Technologies). The I-Cycler was programmed as follows: 94°C for 5 minutes, then 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. Genomic standards, prepared as 20, 100, 500, 2500, and 12500 GE/5µl were prepared and used to generate a standard curve.

DIA data analysis

Threshold Genome Equivalents (GE) values were determined for each of 12 PCR reactions (corresponding to the 1.3kb, 1.8kb, and 2.4kb fragments across the 4 genomic loci) using a previously determined set of cancers and normals. We then applied a requirement that at least 4 of the 12 PCR reactions are above the individual PCR thresholds in order to prospectively determine cancers.

Statistical methods

The impact of sample incubation on DNA recovery, and the impact of stabilization buffer on DNA recovery were both assessed using quantitative-PCR analysis. The data for both comparisons were subjected to a paired-sample t-test. The effect of sample incubation on the integrity of recoverable DNA was analyzed using DIA cut-offs. Resulting DIA scores after incubation were analyzed by Fischer's exact test compared to controls that had been analyzed at t0.

Example 2: The effect of stabilization buffers on DNA integrity assays

The effect of treating a stool sample with a stabilization solution on a subsequent DNA integrity assay (DIA) was examined.

Eight stool samples were removed from a freezer (-80° C) and thawed. To be included in the study the sample was required to contain at least 90 gm of stool. 30 gm aliquots of each thawed sample was then treated for zero hours, 36 hours without TEN and 36 hours with TEN. 30 gm aliquots of some samples were also treated for 24 hours without TEN and 24 hours with TEN.

The 0 hour samples were homogenized immediately with a 7X excess (i.e., (7ml/gm) of TEN buffer (10 mM Tris-C1 pH 8.0, 1 mM EDTA and 150 mM NaCI). The remaining samples were allowed to incubate for 24 hours or 36 hours in the presence or absence of TEN buffer.

At the designated times, 210 ml of TEN buffer was added to the samples that did not already have buffer, and the samples were homogenized and frozen at -80° C. The samples were then prepared and processed for DIA analysis essentially as described in US Patent Application Nos. 20040043467 and 20040014104, and in Boynton et al., Clin. Chem. 49:1058-65, 2003. DNA from four genes (named DIA-D, DIA-E, DIA-X, DIA-Y in Table 2) was captured on a solid substrate using a sequence-specific probe. For each gene, interrogation was performed at distances 0.2, 1.3, 1.8, and 2.4 kb from the capture site using real-time polymerase chain reaction (PCR). Positives were identified by determining genomic equivalents (GE) that were above previously determined cutoffs for the four markers.

The results of the analysis are presented in Table 2. Samples and their time points are grouped together. 24 HR NB represents the 24 hour incubation at room temperature without buffer and the 24 HR B represents the 24 hour incubation at room temperature with buffer. The same is true for the 36 hour designations. The 98% specificity cutoff levels for each locus are indicated at the top of Table 2. A DIA marker may be considered positive if the result is above the 98% cutoff level for that marker (e.g., for one of the markers above 200 bp long). A subject may be considered positive if 3 or more (e.g., 4 or more) DIA markers above 200 bp long are positive (even if two or more of the positive DIA markers are at the same locus). The first two samples 30 and 33 are samples that were negative for DIA making it uninformative for comparing the longer DNA markers with and without buffer. However the 200 markers show a stabilizing effect of the addition of buffer all the way through the 36 hour time point.

The next two samples 36 and 32 are those in which the 0 hour is negative for DIA and the samples with buffer at 36 hours are positive. These are probably borderline samples in which there is a slight difference in GE between the 30 gm aliquots causing one to be positive and the other to be negative. However, a stabilizing effect with the addition of buffer can be seen in the 200 markers and in the few longer markers that do have GE numbers.

The next three samples 29, 24 and 38 are those in which the 0 hour, 36 hour and 24 hour (if available) with buffer were all positive and the 36 hour and 24 hour (if available) without buffer were negative. There is a dramatic difference in the GE numbers in both the short 200 markers and the longer markers between those samples with and without buffer once again demonstrating that the addition of buffer appears to be able to stabilize short and long marker GE numbers up through at least 36 hours. The GE numbers for short and long markers in Sample 31 were stable through 36 hours even in the absence of buffer.

The raw GE numbers were subjected to two different comparisons. First, the GE numbers for each of the markers for samples with buffer were compared to the GE numbers for the same samples at 0 hours. The second comparison was between the GE numbers for each marker for the samples with buffer incubated for 24 or 36 hours at room temperature compared to that same samples without buffer incubated for an equivalent time at room temperature. Before carrying out the calculations some of the GE numbers for some of the samples were discarded because they contained a value of 0 (and so could not be used as the value was used as a divisor in subsequent mathematical manipulations), or it appeared that the capture efficiency was lower for that marker than it was relative to the other markers. After discarding the 0's and outliers the following numbers for the average fold increase in GE numbers by marker for the buffer compared to the 0 hour and the buffer compared to the no buffer were derived.

Table 3. Average fold increase of samples with buffer compared to 0 hour samples.

	DIA-D				DIA-E				DIA-X				DIA-Y			
D100 MuMu	200	1.3	1.8	2.4	200	1.3	1.8	2.4	200	1.3	1.8	2.4	200	1.3	1.8	2.4
1.2	1.6	1.6	1.9	2.1	1.0	1.2	1.0	1.0	0.6	0.9	1.2	0.8	2.5	4.9	4.1	3.0

Table 4. Average fold increase of samples with buffer compared to no buffer samples.

	DIA-D				DIA-E				DIA-X				DIA-Y			
D100 MuMu	200	1.3	1.8	2.4	200	1.3	1.8	2.4	200	1.3	1.8	2.4	200	1.3	1.8	2.4
12.9	39.0	312.8	75.5	1.1	23.7	200.5	231.0	0.8	13.8	71.1	9.1	82.5	89.4	136.9	96.6	195.8

If the addition of buffer was able to stabilize the GE numbers as efficiently as immediate homogenization then the number would be 1 in the first table above. Numbers less than 1 indicate less stability than immediate homogenization, and numbers greater than 1 show a stabilizing effect greater than immediate homogenization. For the most part the numbers are close to 1, indicating that the addition of buffer prior to incubating up to 36 hours has approximately the same effect as immediate homogenization.

For the table in which the samples with buffer were compared to the samples without buffer, the higher the GE number the greater the stabilizing effect the addition of buffer had.

Almost all markers show a dramatic increase in GE numbers with buffer demonstrating that the addition of buffer has a stabilizing effect on the GE numbers for short and long markers. The above data indicate that addition of TEN buffer to stool samples stabilizes the GE numbers for short and long DIA markers in stool samples.

Example 3: The effect of stabilization buffers on DNA integrity and multiple mutation assays

Effect of Sample Handling Conditions on DIA

All samples were frozen (-80°C) within an hour of collection from patients in order to arrest any degradation of DNA in the samples. Subsequently samples were thawed, sectioned into aliquots, and further stored under prescribed conditions. All aliquots were then processed to recover and purify human DNA, and the DNA was analyzed using DIA to assess the impact of the sample incubation conditions on the recovered DNA. DIA has been previously described, and shown to detect long DNA fragments in stool as an epigenetic marker for colorectal cancer (Boynton KA, Summerhayes IC, Ahlquist DA, et al. DNA Integrity as a potential marker for stool-based detection of colorectal cancer. Clin. Chem 2003; 49:7 1058-1065). The DIA analysis consists of real-time PCR reactions for 4 independent genetic loci (designated as D, E, X, and Y), and 3 DNA size markers for each locus, corresponding to approximately 1300, 1800, and 2400 base-pair fragments. Therefore every sample of DNA is subjected to 12 independent analyses that yield a direct measure of the copies of DNA of a specified length in the sample of interest. When a threshold value of copies is exceeded (which is specific to each PCR), a marker is determined to be positive. Similarly, if a minimum of 4 of the markers are positive, the sample is considered to be positive for DIA.

An aliquot of every sample was analyzed immediately after thawing (to). A second aliquot from the same sample was stored at room temperature for a prescribed period of time. Stabilization buffer (210 mins) was added to a third aliquot, and stored for the same period of time. Room temperature incubation times were for > 36 hours. A total of 38 samples were analyzed by DIA. Of the 38 samples, 11 were found to be DIA positive at to. Table 5 shows the detected copy numbers of each DIA marker for these samples, at to, and extended incubation times, with and without stabilization buffer added. The DIA score (number of positive markers per sample) is also indicated. When no stabilization buffer is added, DNA is significantly degraded in 9 of the 11 stool samples when stored for > 36 hours at room temperature. However, for these same samples, addition of stabilization buffer prior to room

temperature incubation yields significantly higher DNA copy number, for all of the DIA markers analyzed. As a result, without addition of buffer, samples that originally were DIA-positive (i.e., at to), become DIA-negative, and with addition of stabilization buffer, the samples remain DIA-positive. Two of the samples (GP-031 and GP-079) yielded high quantities of long DNA fragments even upon extended room temperature incubation without any added stabilization buffer.

In addition, for all of the samples where DIA marker degradation was observed, a loss in total human DNA yield was also observed based on real time quantification of a 200 bp human target sequence (Table 5). As observed with the recovery of DIA markers with buffer addition, total human DNA yields were also recovered by addition of buffer to the stool samples. The remaining 27 samples that were not DIA-positive at to were also divided into aliquots, and incubated with and without stabilization buffer. There was no significant amount of long DNA present in these samples in order to judge the effect of incubation conditions on DNA stability. However, by relying on real-time PCR of shorter fragments (e.g., 200 bp) for the same loci (i.e., D, E, X, and Y) the amount of recoverable human DNA was determined. A sampling of results from this group of specimens is shown in Table 6.

Table 6. Quantification of recoverable human DNA from selected DIA-negative samples, incubated with and without stabilization buffer.

Sample ID	**Incubation conditions**	**D200 (GE/10ul)**	**E200 (GE/10ul)**	**X200 (GE/10ul)**	**Y200 (GE/10ul)**
GP30	none	247	196	418	129
	36h no buffer	50	27	76	0
	36h with buffer	219	216	390	172
GP96	none	129	1120	604	769
	36h no buffer	63	130	100	100
	36h with buffer	34	1270	1140	1290
GP33	none	3690	13200	23300	0
	36h no buffer	61	136	422	103
	36h with buffer	2220	4640	3860	2

GP125	none	21	105	81	74
	36h no buffer	47	93	68	65
	36h with buffer	38	68	25	29
LSP49-20	OHR	359	95	91	202
	48HR NB	148	204	101	188
	48HR B	310	127	101	411
LSP20-21	none	1140	807	1500	337
	36h no buffer	219	588	421	285
	36h with buffer	542	363	174	591

Similar trends were found for the DIA-negative samples as with the DIA-positive samples. Without any buffer added, the majority of samples (16/27; 59%) stored at room temperature (either 36 or 48 hours) prior to recovery of the DNA, showed significant decrease in human DNA yield, as judged by a direct comparison of the copy number of short fragments compared to the *to* measurement. The majority of samples (22/27; 81%) were preserved upon addition of stabilization buffer to the stool sample (for example samples GP30 and GP96, in Table 6). In the case of five samples, the addition of stabilization buffer did not offer any significant advantage in recoverable DNA, compared to samples stored without buffer (see for example, LSP20-21 in Table 6).

Effect of Sample Handling Conditions on Gene Mutation Markers

It has previously been shown that DNA recovered from stool can be interrogated for specific mutations known to be associated with colorectal cancer (CRC) (Ahlquist DA, Skoletsky JE, Boynton KA, et al. Colorectal cancer screening by detection of altered human DNA in stool: Feasibility of a multi-target assay panel. Gastroenterology 2000; 119:1219-1227; Brand RE, Ross ME, Shuber AP. Reproducibility of a Multi-Target Stool-Based Assay for Colorectal Cancer Detection. Am J Gastroenterology (in press); Tagore KS, Lawson MJ, Yucaitis JA, et al. Sensitivity and Specificity of a Stool DNA Multi-Target Assay Panel for the Detection of Advanced Colorectal Neoplasia. Clinical Colorectal Cancer 2003; 3 (1), 47-53). In the experiments described above it was shown that upon room temperature incubation of

stool samples long fragments of DNA are degraded, significantly diminishing the usefulness of epigenetic DIA markers. Further it was shown that human DNA yield is reduced introducing the question of whether or not sufficient amount of DNA template molecules remain for point mutation analysis in known CRC associated genes (e.g., *Kras, APC,* and *p53).* Stool samples from confirmed CRC patients were collected and immediately frozen. All 11 samples were confirmed to contain one or more point mutations, as shown in Table 7.

Table 7. Gene mutation analysis results for stool aliquots incubated at room temperature.

Sample ID	Incubation time	DNA Recovery (GE/10ul)	Marker (Gene; Condon)	SBE Threshold	SBE signal	
					1st pass	2nd pass
GP-003	*to*	2,180	*Kras; kl2p2*	0.400	0.445	1.290
	72h	351			1.504	
	to		*p53; 273p1*	0.200	0.999	2.419
	72h				1.705	
GP-023	*to*	59,800	*Kras; kl2p2*	0.400	1.071	1.250
	72h	3,250			2.423	
GP-024	*to*	509	*BAT-26*	0.050	0.093	
	48h	119			0.380	
	72h	217			0.092	
GP-025	*to*	788	*p53; 248p2*	1.000	6.648	4.666
	48h	121			1.206	
	72h	125			3.697	
GP-026	*to*	458	*p53; 175p2*	0.400	1.459	1.459
	48h	160			1.914	1.293
	72h	185			0.449	1.815
GP-034	*to*	72,300	*p53;248p2*	1.000	1.505	1.296
	36h	1,180			4.834	
GP-029	*to*	10,700	*p53; 175p2*	0.400	1.229	1.534
	36h	1,110			1.721	1.641
	36h, Buffer	11,000			1.793	1.459
GP-030	*to*	501	*Kras; kl3p2*	0.400	1.814	4.203
	36h	135			3.650	
	36h, Buffer	338			2.593	
GP-031	*to*	10,300	*APC; 1309*	0.055	0.219	0.169
	36h	13,500			0.090	
	36h, Buffer	4,550			0.210	

GP-105	*to*	1,540	*APC; 1554*	0.250	1.210	1.119
	48h	78			**0.049**	**0.010**
	48h, Buffer	9,140			2.392	0.466
GP-121	*to*	4,720	*p53; 175p2*	0.400	2.699	3.626
	48h	1,500			1.601	1.181
	48h, Buffer	1,040			2.329	3.628

Bold = negative mutation score

Human DNA yield in the sample is judged from the quantifiable recovery of DNA from aliquots stored under the different conditions (Table 7). Without buffer added to the samples, on average only 17% of the DNA in each sample (excluding sample GP-031) was recovered after incubation of samples at room temperature for > 36 hours. The one exception, sample GP-031, maintained high DNA yield even without addition of stabilization buffer. Also, as with the DIA experiments, samples incubated with stabilization buffer maintained human DNA yields similar to the t_o samples.

Aliquots from all samples were analyzed for mutations initially (t_o), and additional aliquots were analyzed after prescribed room temperature incubation times. Aliquots from the first 6 stool samples (in Table 7) were simply stored at room temperature with no buffer added, whereas aliquots from the next set of 5 samples were stored with and without the addition of stabilization buffer. The SBE signal values listed in Table 7 are derived from a ratio of the CE peak intensity for a mutant specific product, to the peak intensity of an internal control (see Materials and Methods). In this context the values are a crude measure of the percent mutant DNA in the sample (which is typically 1-5%), and must exceed a minimum value (SBE threshold which is specific to each marker) to be judged as positive (i.e., significantly above background). Interestingly, although DNA was degraded for 10 of 11 samples stored at room temperature without buffer added, mutations were reproducibly detected for 9 of the 10, as well as for GP-031 (which did not show degradation of DNA). Sample GP-105, by contrast, was originally shown to contain an *APC* mutation (at codon 1554), and after incubation of an aliquot for 48 hours without buffer, the mutation was no longer detectable even with repeated analysis. This sample demonstrated a significant reduction in human DNA yield, with only 5% of the original value recovered after room temperature incubation. However, when GP-105 was incubated in the presence of stabilization buffer, the originally identified mutation was detected. In addition, as we observed within the DIA marker experiments, human DNA

recovery for all samples remained high when incubated in the presence of stabilization buffer.

Analysis of Results for DNA Integrity Assays and Gene Mutation Markers

All samples were initially frozen within 1 hour of collection, and then stored frozen until needed. In the first set of experiments, aliquots from 38 samples were analyzed to determine the effect of different sample handling conditions on the DIA marker. Eleven samples were found to be DIA-positive when analyzed immediately after thawing, indicating that long DNA fragments were recovered and amplified from the stool sample. Based on a previously established algorithm (described above), 9 samples went from being DIA-positive to DIA-negative when incubated at room temperature with no buffer added, due to a significant loss of long DNA copies. The loss in long fragments was consistent for each of the loci (D, E, X, and Y) interrogated. In addition, when analyzed for the total yield of human DNA from these samples (i.e., including the shortest fragment size of 200bp), they all demonstrated a significant loss in recovery. Two of the 11 samples remained DIA-positive even after storing at room temperature > 36 hours without stabilization buffer added. When stabilization buffer is added to samples prior to room temperature incubation all of the DIA-positive samples (11/11; 100%) remain positive after >36 hours. We observed some degree of variation in recovery of long fragments. However the variation is not significant with respect to DIA scoring of DNA recovery.

Similar trends were observed relative to the reduction of recoverable human DNA for the 27 samples that were initially DIA-negative. Human DNA recovery was significantly reduced for the majority of samples (25/27; 93%), and only 2 were stable when incubated at room temperature without buffer added. Upon addition of stabilization buffer to stool samples prior to room temperature incubation, human DNA yield was maintained in 81% (22/27) of the samples. There was no apparent benefit upon addition of buffer for 5 samples, whereas with DIA-positive samples all (11/11) benefited, in terms of DNA integrity or human DNA yield. This difference is likely linked to the fact that the DIA-negative samples contained less recoverable human DNA overall. A DNA recovery value for each sample was calculated by averaging the recovery values for all 4 loci of the 200bp PCR for each sample. The median recovery for the DIA-negative samples was 452 genome equivalents (GE), and 8,410 for the DIA-positive samples ($P<0.0001$). Overall, this suggests that DIA-positive samples have almost 20-fold more human DNA recoverable from stool. Without addition of stabilization buffer these samples might otherwise become false-negatives under adverse incubation

conditions.

Results of the matched aliquots with and without stabilization buffer were first subjected to statistical analysis without any assumptions as to the mechanism of action with the buffer. Using a one-tailed paired t-test, we found a significant decrease ($P = 0.0018$) in recovered DNA, without addition of buffer, for aliquots that had been incubated at room temperature (control = *to*). The analysis was based on quantitative PCR data for the shortest DNA fragment size (i.e., 200bp) for all samples. Likewise the addition of buffer to samples prior to room temperature incubation was found to yield a significant increase in DNA recovery, relative to the matched aliquots with no buffer added ($P = 0.010$). Finally, in order to determine the significance of the addition of stabilization buffer on DIA scores (and therefore the integrity of the DNA), results were subjected to a Fischer's exact test, analyzing the samples that were found to be DIA positive at *to* ($N = 11$). Addition of buffer significantly preserves DNA integrity ($P = 0.0002$) compared to aliquots with no buffer addition. Therefore, whereas 9 of 11 samples became DIA-negative after incubation at room temperature when no buffer was added, buffer addition led to a recovery of the 9 samples, such that even after 36h incubation all of the samples (11 of 11) were positive.

In order to determine the effect of DNA degradation on stool DNA mutation detection, analyses were performed on stool samples that were informative for specific DNA associated point mutations. In the second set of experiments, 11 samples were determined to be positive by at least one point mutation marker in a mutation panel, and the samples were then re-analyzed with the mutation panel after sample aliquots had been incubated under the same incubation conditions as described above. Without addition of stabilization buffer, the recoverable DNA in 10 of 11 samples was significantly diminished. On average, for these samples, 83% of the original DNA was lost. Only one sample did not demonstrate a loss in human DNA yield upon room temperature incubation. Despite the loss of DNA, the original mutations could be confirmed after room temperature incubation in 10 of 11 samples (91%). As an example, one sample had 2180 GE of recoverable human DNA at *to*, and was originally found to be positive for a *Kras* and *a p53* marker. Upon room temperature incubation of the stool aliquot for 72 hours, only 351 GE of amplifiable DNA was recovered, yet the sample remained positive for both markers. One sample, in addition to having a significant reduction in human DNA yield, also had a loss of an *APC* mutation originally detected at *to*. However, with stabilization buffer added, a high yield of human DNA was measured after 48 hrs of incubation, and the sample remained positive for the APC mutation.

Results from these experiments indicate that DIA is quite sensitive to sample incubation conditions, and DNA degradation, whereas the mutation panel is more refractory. In one aspect of the invention, incubation of samples at room temperature for 36 hours or more in the absence of stabilization buffer may degrade DNA to the point that any remaining long fragments fall below an DNA integrity assay cut-off. A point mutation assay, on the other hand, is dependent on the detection of mutant DNA in the presence of wild-type, where the percent mutant is often only on the order of 1% of the total human DNA, and PCR amplification efficiency is not dependent on the size of the template molecules. Therefore even in the case of adverse incubation conditions, as long as at least a single copy of mutant sequence is recovered and amplified in the PCR reactions, it should be possible to detect mutations downstream in the analytical process. Interestingly, of the mutation positive samples that had been stored without stabilization buffer, the one where the mutation was lost had the lowest measurable DNA, suggesting that the lack of detection was likely due to an insufficient number of mutant molecules to yield a signal above background. In addition, even though the remaining mutation positive samples remained positive with or without buffer, a consistent loss of human DNA yield was observed in all samples when stabilization buffer was not added.

According to aspects of the invention, despite the stability of a point mutation analysis, it may be desirable to mitigate degradation of DNA, particularly when the amount of abnormal DNA in a sample may be very low. According to the invention, in order to maximize clinical sensitivity of fecal DNA assays it is important to maximize the recovery of otherwise scarce amounts of human DNA. Accordingly, aspects of the invention may be particularly useful for detecting indicia of adenoma and/or early stage cancer. In one embodiment, the invention may be useful to avoid or reduce the number of false negative results in a screen of a population of individuals for one or more indicia of adenoma or early stage cancer (e.g., using a DNA integrity assay, a multiple mutation assay, a hypermethylation assay, or any combination thereof).

In summary, under adverse sample handling conditions, 91% of samples (10/11; 95%CI: 59 - 100%) that were positive by the point mutation panel remain positive. Under the same conditions, however, only 18% of samples (2/11; 95%CI: 2 - 52%) that were DIA-positive when analyzed fresh, remained positive after room temperature incubation. Transporting samples frozen is a viable means of temporarily neutralizing active nucleases and mitigating degradation of DNA. However, insuring that samples are frozen immediately, and

keeping samples frozen during transport to test-facilities poses challenges and risks in a population based screening application of fecal DNA assays. According to the invention, an alternative is to add a stabilization buffer to the sample as a way of preserving DNA such that samples could be stored (or transported) at room temperature. The results of these experiments show that all DIA-positive samples (11/11; 100%), and all mutation panel positive samples (11/11; 100%) remained positive after storing for >36 hours after addition of stabilization buffer to the stool.

In any of the aspects of the invention described herein, including the examples, a sample (e.g., a stool sample) may be directly deposited into a container (e.g., a sealable conatiner) that already contains stabilization solution. The amount of solution may be sufficient to cover the sample or a portion thereof. Alternatively, stabilization solution may be added to a container when (e.g., at the same time, immediately after, or within minutes, hours, or a day) a biological sample (e.g., a stool sample) is deposited in the container. The amount of solution added may be sufficient to cover the entire sample or at least a portion thereof (e.g., 10%, 25%, 50%, or more). In one embodiment, an amount of solution sufficient to fill the container may be added. In some embodiment, a container may be between about 25 mls and about 1,000 mls (e.g., between about 50 mls and about 500 mls, between about 100 mls and about 400 mls, or about 250 mls). However, smaller or larger containers may be used. A container with sample and stabilization solution may be sealed for storage/shipping.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

CLAIMS

1. A method for preparing a nucleic acid for analysis, the method comprising:

providing a patient sample comprising a nucleic acid and shed cells or cellular debris; and

contacting said patient sample with a stabilization solution under conditions sufficient to stabilize said nucleic acid for nucleic acid integrity analysis and/or multiple mutation analysis, wherein said stabilization solution comprises a buffer, a salt, and a chelating agent, thereby preparing a nucleic acid for a nucleic acid integrity analysis.

2. The method of claim 1, wherein the conditions are sufficient to detect at least a three-fold genomic equivalent (GE) increase in a nucleic acid integrity analysis of a patient sample having adenoma or cancer as compared to the GE detected in a nucleic acid integrity analysis of a sample from said patient that is not incubated with said stabilization solution.

3. The method of claim 1, wherein the integrity analysis is performed by determining an amount of nucleic acid greater than about 200 nucleotides in length using an assay that detects wild-type or mutant nucleic acid, wherein said nucleic acid is present in a patient sample comprising shed cells or cellular debris; and identifying said patient as having cancer or adenoma if said amount is greater than an amount of nucleic acid expected to be present in a sample obtained from a patient who does not have cancer or adenoma.

4. The method of claim 1, wherein the patient sample is a sample from a human.

5. The method of claim 1, wherein the patient sample is a stool sample.

6. The method of claim 3, wherein the patient sample is a stool sample.

7. The method of claim 1, wherein the patient sample is frozen and thawed prior to incubation with stabilization solution.

8. The method of claim 1, wherein the patient sample is not frozen prior to incubation with said stabilization solution.

9. The method of claim 1, wherein the stabilization solution is present at 1-7 ml per gram of patient sample.

10. The method of claim 1, wherein the patient sample and stabilization solution are incubated at about 17 to 28 degrees Centigrade.

11. The method of claim I, wherein the patient sample and stabilization solution are incubated at about 20 to 25 degrees Centigrade.

12. The method of claim 1, wherein the patient sample and stabilization solution are incubated at least 6 hours.

13. The method of claim 1, wherein the patient sample and stabilization solution are incubated at least 12 hours.

14. The method of claim 1, wherein the patient sample and stabilization solution are incubated at least 24 hours.

15. The method of claim 1, wherein the patient sample and stabilization solution are incubated at least 36 hours.

16. The method of claim 1, wherein the buffer in said stabilization solution is 0.5 mM to 25 mM Tris.

17. The method of claim 1, wherein the buffer in said stabilization solution is 5 mM to 15 mM Tris.

18. The method of claim 1, wherein the buffer in said stabilization solution is 8 mM to 13mMTris.

19. The method of claim 1, wherein the buffer in said stabilization solution is 10 mM Tris.

20. The method of claim 1, wherein the chelating agent in said stabilization solution is 0.01 to 2.5 mM EDTA.

21. The method of claim 1, wherein the chelating agent in said stabilization solution is .75-1.25 mM EDTA.

22. The method of claim 1, wherein the chelating agent in said stabilization solution is 1 mM EDTA.

23. The method of claim 1, wherein the salt in said stabilization solution is 75 mM to 225 mM NaCl.

24. The method of claim 1, wherein the salt in said stabilization solution salt in stabilization solution is 100 mM to 175 mM NaCl.

25. The method of claim 1, wherein the salt in said stabilization solution salt in stabilization solution is 150 mM NaCl.

26. The method of claim 1, wherein said stabilization solution is provided at pH 7.0 to 9.0.

27. The method of claim 1, wherein said stabilization solution is provided at pH 7.5 to 8.5.

28. The method of claim 1, wherein said stabilization solution is provided at about pH 8.0.

29. The method of claim 1, further comprising:

determining in said incubated patient sample an amount of nucleic acid greater than about 200 bp in length using an assay that detects wild-type or mutant nucleic acid; and

identifying said patient as having cancer or adenoma if said amount is greater than an amount of nucleic acid expected to be present in a sample obtained from a patient who does not have cancer or adenoma.

30. A method for preparing a nucleic acid for an assay for diagnosing a carcinoma or adenoma, the method comprising:

providing a patient stool sample comprising shed cells or cellular debris and a nucleic acid; and

contacting said patient sample with a stabilization solution under conditions sufficient to stabilize said nucleic acid for nucleic acid integrity and/or multiple mutation analysis, wherein said stabilization solution is about pH 7.5 to about pH 8.5 and comprises 0.5 mM to 25 mM Tris, 0.01 to 2.5 mM EDTA and 100 mM to 200 mM NaCl, and wherein the conditions are sufficient to detect at least a three-fold genomic equivalent (GE) increase in a nucleic acid integrity analysis of a patient sample having adenoma or cancer as compared to the GE detected in a nucleic acid integrity analysis of a sample from said patient that is not incubated with said stabilization solution, thereby preparing a nucleic acid for a nucleic acid integrity analysis.

TABLE 2

			98% Specificity		0	80	30	27	0	60	45	18	0	27	17	8	0	60	30	15	
					DIA-D				DIA-E				DIA-X				DIA-Y				
Sample	Diagnosis	Morphology	TIME POINT	D100 Mu Mu	200	1.3	1.8	2.4	200	1.3	1.8	2.4	200	1.3	1.8	2.4	200	1.3	1.8	2.4	LcDIA Score
GP30A	Cancer	Liquid	0 Hr	482	247	36	47	21	196	76	43	28	418	4	5	6	129	6	12	7	3
GP30B			24 Hr NB	246	51	0	0	0	54	0	4	0	115	1	1	1	50	1	1	3	0
GP30C			24 Hr B	601	159	0	0	0	163	15	7	0	315	2	0	1	101	1	1	3	0
GP30D			36 Hr NB	118	50	0	13	0	27	7	0	0	76	3	3	2	0	0	1	1	0
GP30E			36 Hr B	538	219	0	23	0	216	20	11	0	390	11	10	12	172	2	8	1	1
GP33A	Cancer	Semi-Liquid	0 Hr	32000	3690	6	14	0	13200	172	63	44	23300	4	6	3	0	0	0	0	3
GP33D			36 Hr NB	606	61	0	0	0	136	0	0	0	422	0	0	1	103	2	3	3	0
GP33E			36 Hr B	17100	2220	9	57	0	4640	43	6	0	3860	1	1	1	2	1	1	2	1
GP36A	Control	Normal	0 Hr	450	118	0	0	0	180	28	27	0	56	1	0	1	31	0	1	1	0
GP36D			36 Hr NB	212	0	0	0	0	54	0	0	0	19	1	0	1	24	1	1	1	0
GP36E			36 Hr B	988	258	62	29	28	499	156	176	53	6	1	0	0	185	6	9	9	4
GP32A	Cancer	Liquid	0 Hr	21300	4690	15	0	0	3830	126	34	37	14000	15	15	18	4040	2	1	4	3
GP32D			36 Hr NB	4150	1160	0	18	14	752	38	20	14	309	1	0	1	1490	9	21	6	0
GP32E			36 Hr B	38700	14700	55	52	27	2460	69	15	19	7720	16	21	26	15000	13	14	15	7
GP29A	Cancer	Liquid	0 Hr	7830	2120	394	522	150	3340	1490	734	343	5560	141	98	118	9	1	3	2	9
GP29B			24 Hr NB	1200	92	0	0	0	161	0	0	0	505	1	0	0	140	11	8	17	1
GP29C			24 Hr B	13500	2380	812	1490	603	3720	2030	1090	710	6230	202	476	198	2890	150	236	247	12
GP29D			36 Hr NB	1470	131	0	0	0	324	1230	0	0	1010	2	1	2	8	5	12	8	1
GP29E			36 Hr B	9410	58	10	28	21	2770	1170	1050	768	2750	105	52	53	11	1	2	3	6
GP34A	Cancer	Semi-Liquid	0 Hr	56000	15800	1180	840	318	34600	4890	3160	2180	36100	247	387	299	293	1	1	2	9
GP34B			24 Hr NB	2870	672	10	13	0	951	13	11	0	3600	5	4	6	571	6	7	9	0
GP34C			24 Hr B	111000	68200	6170	3890	2230	72700	11800	8960	7070	193	18	12	12	61400	2450	1620	4930	10
GP34D			36 Hr NB	1570	279	0	0	0	471	17	6	0	517	2	0	2	77	2	2	2	0
GP34E			36 Hr B	43900	29400	1250	2090	959	31600	7650	3090	2760	27400	366	516	353	24000	859	577	1720	12
GP38A	Control	Normal	0 Hr	66300	35500	735	598	202	46400	3930	2300	3000	28	2	1	1	16700	114	103	243	9
GP38D			36 Hr NB	1750	103	0	0	0	375	22	9	0	284	1	2	1	47	0	0	0	0
GP38E			36 Hr B	13400	6320	88	81	35	10300	426	369	310	7510	4	6	5	7930	39	19	26	7
GP31A	Cancer	Normal	0 Hr	9270	4100	200	223	134	3290	992	493	250	5960	230	125	108	2510	47	36	44	11
GP31D			36 Hr NB	12900	7220	274	441	205	6390	1320	573	241	3060	66	45	111	3230	52	79	41	11
GP31E			36 Hr B	6800	3400	129	140	56	1080	257	116	41	4990	30	24	22	ND	ND	ND	ND	9

ND = Not Done

TABLE 5 – Results of DNA integrity assay for DIA-positive samples incubated at room temperature for prescribed times, with and without stabilization buffer added. = DIA- positive marker; = DIA-positive sample

		DIA-D				DIA-E				DIA-X				DIA-Y				
SAMPLE	INCUBATION	200bp	1.3kb	1.8kb	2.4kb	200bp	1.3kb	1.8kb	2.4kb	200bp	1.3kb	1.8kb	2.4kb	200bp	1.3kb	1.8kb	2.4kb	LcDIA Score
GP32	0 HR	4690	15	0	0	3830	126	34	37	14000	15	15	18	4040	2	1	4	3
	36 HR	1160	0	18	14	752	38	20	14	309	1	0	1	1490	9	21	6	0
	36 HR, Buffer	14700	55	52	27	2460	69	15	19	7720	16	21	26	15000	13	14	15	7
GP29	0 HR	2120	394	522	150	3340	1490	734	343	5560	141	98	118	9	1	3	2	9
	36 HR	131	0	0	0	324	1230	0	0	1010	2	1	2	8	5	12	8	1
	36 HR, Buffer	58	10	26	21	2770	1170	1050	768	2750	105	52	53	11	1	2	3	6
GP34	0 HR	15800	1180	840	318	34600	4890	3160	2180	36100	247	387	299	293	1	1	2	9
	36 HR	279	0	0	0	471	17	6	0	517	2	0	2	77	2	2	2	0
	36 HR, Buffer	29400	1250	2090	959	31600	7650	3090	2760	27400	366	516	353	24000	859	577	1720	12
GP38	0 HR	35500	735	598	202	46400	3930	2300	3000	28	2	1	1	16700	114	103	243	9
	36 HR	103	0	0	0	375	22	9	0	284	1	2	1	47	0	0	0	0
	36 HR, Buffer	6320	88	81	35	10300	426	369	310	7510	4	6	5	7930	39	19	26	7
GP31	0 HR	4100	200	223	134	3290	992	493	250	5960	230	125	108	2510	47	36	44	11
	36 HR	7220	274	441	205	6390	1320	573	241	3060	66	45	111	3230	52	79	41	11
	36 HR, Buffer	3400	129	140	56	1080	257	116	41	4990	30	24	22	ND	ND	ND	ND	12
107	0 HR	17900	672	1310	236	6890	1020	813	471	14900	1770	137	138	5970	1060	908	605	12
	48 HR	23	0	0	0	21	5	4	0	15	1	1	4	23	3	2	220	1
	48 HR, Buffer	3600	567	1120	294	1860	647	541	308	3710	1110	101	89	1790	615	778	576	12
117	0 HR	6230	170	519	78	2620	320	130	108	3340	255	24	15	2830	453	357	312	12
	48 HR	4	0	0	0	4	0	0	0	3	1	1	2	3	0	0	0	0
	48 HR, Buffer	12100	1170	7590	755	7120	3840	3000	2810	9020	4490	3790	4860	3770	1930	1640	1540	12
97	0 HR	2790	36	202	10	2100	78	33	10	2260	15	5	4	2090	79	123	43	5
	48 HR	16	0	0	0	12	0	3	0	33	2	0	3	22	1	1	0	0
	48 HR, Buffer	1400	50	84	22	1180	116	63	26	1920	45	16	34	1690	141	121	123	9
105	0 HR	1690	71	233	47	1420	258	181	83	1660	123	18	11	1320	167	120	315	11
	48 HR	24	0	61	0	30	0	2	0	13	1	0	1	29	1	2	0	1
	48 HR, Buffer	379	29	118	9	549	81	50	33	469	18	2	2	626	67	44	46	7
90	0 HR	36000	469	1560	167	1070	86	26	96	15900	942	65	91	9520	1420	890	1380	11
	48 HR	175	0	0	0	244	4	6	0	54	1	0	1	198	4	2	8	0
	48 HR, Buffer	72900	1320	8290	398	30900	13500	4870	4600	22700	2490	183	67	10500	2420	1790	6270	12
GP79	0 HR	4470	250	21	21	20400	1250	107	124	12200	44	62	52	13200	392	728	0	9
	48 HR	3680	72	20	11	14600	528	53	64	9790	21	27	22	6370	192	370	676	8
	48 HR, Buffer	3980	128	70	24	14100	446	89	87	8510	35	51	36	8190	241	663	0	10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/017046

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/61808 A (EXACT LABORATORIES, INC) 19 October 2000 (2000-10-19) pages 11-15; example 1	1-30
X	WO 00/50640 A (EXACT LABORATORIES, INC) 31 August 2000 (2000-08-31) pages 15-16	1-30
A	SAMBROOK J ET AL: "MOLECULAR CLONING. LABORATORY MANUAL" 2001, COLD SPRING HARBOR, NY : CSHL PRESS, US, PAGE A 1.22 , NEW YORK , XP002345193 the whole document	1-30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 2005	10/10/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Reuter, U

3

Form PCT/ISA/210 (second sheet) (January 2004)

Geneoscopy Exhibit 1009, Page 37

Exact Sciences Ex. 2005, Page 185 of 760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/017046

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-30 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the part of the method that does not comprise a step of providing a sample that is practised on the human/animal body.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**01/16/2024 07:00:05 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	63959858	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 71

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Ex_1020-Nonfinal OA for US Appl 17936335.pdf	7	Other reference-Patent/Application/Search Documents	477 KB
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2024-01-16_35239.307_PetitionWithdrawIssue.pdf	2	Petition to Withdraw from Issue	162 KB
2024-01-16_35239.307_IDS.pdf	9	Information Disclosure Statement (IDS) Form (SB08)	363 KB

Warning: This is not a USPTO supplied IDS fillable form. Data in the form cannot be automatically loaded to other USPTO systems. The attached file contains comments or annotations. Comments are not recommended and may cause processing problems with the document.

2024-01-16_35239.307_RCE.pdf	3	Request for Continued Examination (RCE)	131 KB

Warning: This is not a USPTO supplied RCE fillable form. Data in the form cannot be automatically loaded to other USPTO systems.

2023-12-04_ReExam Certificate.pdf	2	Other reference-Patent/Application/Search Documents	44 KB

ZOU_2007_NPL.pdf	11	Non Patent Literature	8644 KB
Petition for IPR.pdf	87	Other reference-Patent/Application/Search Documents	1124 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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Ex_1020-Nonfinal OA for US Appl 17936335.pdf	27E5FA6779FB96F5FD95F664000EDC0F6CDFF4C4ED9E38F65B790DCFC5824033F8D24BC310E94795B93A1D1BC72574A9E7C240DBB47D7D7AA2CBF79C4A5D2893
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	18179945	Filing Date	2023-03-07	Docket Number (if applicable)	EXCTD-35239.307	Art Unit	1634
First Named Inventor	LOUWAGIE, Joost			Examiner Name	WHISENANT		

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

SUBMISSION REQUIRED UNDER 37 CFR 1.114

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

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

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

☐ Other _____

☒ Enclosed

☐ Amendment/Reply

☒ Information Disclosure Statement (IDS)

☐ Affidavit(s)/ Declaration(s)

☒ Other
Petition to Withdraw from Issue

MISCELLANEOUS

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

☐ Other _____

FEES

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

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(54) Title: EPIGENETIC CHANGE IN SELECTED GENES AND CANCER

(57) 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, JPII3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXEl, SYNEl, S0X17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, cancer. Also described are pharmacogenetic methods for determining suitable treatment regimens for cancer and methods for treating cancer patients, based around selection of the patients according to the methods of the invention. The present invention is also concerned with improved methods of collecting, processing and analyzing samples, in particular body fluid samples. These methods may be useful in diagnosing, staging or otherwise characterizing various diseases. The invention also relates to methods for identifying, diagnosing, staging or otherwise characterizing cancers, in particular gastrointestinal cancers such as colorectal cancers, gastric cancers and oesophageal cancers. The methods of the invention relate, inter alia, to isolating and analyzing the human DNA component from faecal samples and blood-based samples.

WO 2008/084219 A1

EPIGENETIC CHANGE IN SELECTED GENES AND CANCER

FIELD OF THE INVENTION

The present invention relates to methods and kits for identifying and diagnosing cancer which include detecting an epigenetic change, such as a change in the methylation status, or the expression levels, or a combination thereof of any one or more of a number of genes. Also described are pharmacogenetic methods for determining suitable treatment regimens for cancer and methods for treating cancer patients, based around selection of the patients according to the methods of the invention. The present invention is also concerned with improved methods of collecting, processing and analyzing samples, in particular body fluid samples. More particularly, the invention relates to methods for identifying epigenetic changes in body fluid samples. These methods may be useful in diagnosing, staging or otherwise characterizing various diseases. The invention also relates to methods for identifying, diagnosing, staging or otherwise characterizing cancers, in particular gastrointestinal cancers such as colorectal cancers, gastric cancers and oesophageal cancers. The methods of the invention relate, *inter alia*, to isolating and analyzing the human DNA component from faecal samples and blood-based samples.

BACKGROUND OF THE INVENTION

In their earliest stages most cancers are clinically silent. Patient diagnosis typically involves invasive procedures that frequently lack sensitivity and accuracy. Highly reliable, non-invasive screening methods would permit easier patient screening, diagnosis and prognostic evaluation.

Tumour derived markers are biological substances that are usually produced by malignant tumours. Ideally a tumour derived marker should be tumour-specific, provide an indication of tumour burden and should be produced in sufficient amounts to allow the detection of minimal disease. Most tumour derived markers used in clinical practice are tumour antigens, enzymes, hormones, receptors and growth factors that are detected by biochemical assays. The detection of DNA alterations such as mutations, deletions and epigenetic modifications (Baylin et al., 2000) provide another means for identifying cancers.

An epigenetic modification can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, mediated by mechanisms other than alterations in the primary nucleotide sequence of a gene. It is now general knowledge that both genetic and epigenetic alterations can lead to gene silencing and cellular dysfunction. Synergy between these two processes drives tumor progression and malignancy. Three related mechanisms that cause alteration in gene expression are recognised: DNA methylation, histone code changes and RNA interference.

DNA hypermethylation is an epigenetic modification whereby the gene activity is controlled by adding methyl groups (CH_3) to specific cytosines of the DNA. In particular, methylation occurs in the cytosine of the CpG dinucleotides (CpG islands) which are concentrated in the promoter regions and introns in human genes (P.A. Jones et al., 2002; P.W. Laird et al., 2003). Methylation is associated with gene

silencing. DNA hypermethylation is found to be involved in a variety of cancers including lung, breast, ovarian, kidney, cervical, prostate and also colorectal cancer. Methylation patterns of DNA from cancer cells are significantly different from those of normal cells. Therefore, detection of methylation patterns in appropriately selected genes of cancer cells can lead to discrimination of cancer cells from normal cells, thereby providing an approach to early detection of cancer.

DNA tumour markers, in particular DNA methylation markers, offer certain advantages when compared to other biochemical markers. An important advantage is that DNA alterations often precede apparent malignant changes and thus may be of use in early diagnosis of cancer. Since DNA is much more stable and, unlike protein, can be amplified by powerful amplification-based techniques for increased sensitivity, it offers applicability for situations where sensitive detection is necessary, such as when tumour DNA is scarce or diluted by an excess of normal DNA (Sidransky et al.,1997). Bodily fluids provide a cost-effective and early non-invasive procedure for cancer detection. In this context, faecal-based cancer testing has been one area of investigation.

Human colorectal cancer has provided a good model for investigating whether DNA cancer markers can be adopted as an optimal faecal-based diagnostic screening test. Central to faecal-based colorectal cancer testing has been the identification of specific and sensitive cancer derived markers.

The N-Myc downstream-regulated gene (NDRG) family comprises four family members: NDRG1 (NDRG-family member 1), NDRG2 (NDRG-family member 2), NDRG3 (NDRG-family member 3) and NDRG4 (NDRG-family member 4). The human NDRG1 and NDRG3 belong to one subfamily, and NDRG2 and NDRG4 to another. At amino acid (aa) level, the four members share 53-65% identity. The four proteins contain an alpha/beta hydrolase fold as in human lysosomal acid lipase but are suggested to display different specific functions in distinct tissues.

NDRG1 codes for a cytoplasmic protein believed to be involved in stress responses, hormone responses, cell growth, and cell differentiation. NDRG1 has been demonstrated to be upregulated during cell differentiation, repressed by N-myc and c-myc in embryonic cells, and suppressed in several tumor cells (Qu X *et al.*,2002; Guan *et al.*, 2000).

NDRG3 is believed to play a role in spermatogenesis since it is highly expressed in testis, prostate and ovary (Zhao W *et al.*, 2001). Its involvement in brain cancer development has also been suggested (Qu X *et al. 2002*).

NDRG2 codes for a cytoplasmic protein that seems to be involved in neurite outgrowth and in glioblastoma carcinogenesis (Deng Y *et al., 2003)*. It is upregulated at both the RNA and protein levels in Alzheimer's disease brains (Mitchelmore C *et al., 2004)*, and has also been suggested to play an important role in the development of brain cancer (Qu X *et al. 2002*), pancreatic cancer and liver cancer (Hu XL *et al.*, 2004).

The NDRG4 cytoplasmic protein is involved in the regulation of mitogenic signalling in vascular smooth muscles cells (Nishimoto S *et al.*). The NDRG4 gene contains 17 exons, and several alternatively spliced transcript variants of this gene have been described. NDRG4 may also be involved in brain cancer development (Qu X *et al. 2002*).

Suppressed expression of NDRG-family genes has been demonstrated in a number of tumours (Qu X *et al. 2002*) and the involvement of DNA promoter hypermethylation is limited to the reporting of NDRG2 methylation in brain tumors (Lusis *et al.*, 2005).

Initially, faecal-based DNA assays investigated the usefulness of specific point mutations markers for detecting colorectal cancer. Later, the DNA integrity in faecal samples proved to be a useful marker (Boynton et al., 2003). Finally, faecal testing based on DNA alterations gradually evolved into the development of a multi-target DNA assay using specific point mutation markers, a microsatellite instability marker and a marker for DNA integrity. Recently, the potential of faecal DNA testing targeting epigenetic alterations has been investigated (Müller et al., 2004, Chen et al., 2005) and has been added to the multi-target DNA assay. Genes having an altered methylation status traceable in faecal DNA from colon cancer patients versus control samples from healthy subjects have been discovered (Belshaw et al., 2004; Petko et al.,2005; Lenhard et al.,2005; Müller et al.,2004; Chen et al., 2005 and Lueng et al., 2004).

Factors that may influence the sensitivity of the selected markers are sampling processing procedures and DNA isolation and extraction protocols. One challenge faced by researchers investigating colorectal cancer is the diversity of DNA present in stool samples. Most of the DNA recovered from faecal samples is bacterial in origin, with the human DNA component representing only a very small minority. Human DNA from cells sloughed from the colonic mucosa represents as little as 0.1 to 0.01% of the total DNA recoverable from stool. Additionally, the human DNA recovered is highly heterogeneous. Normal cells are sloughed into the colonic lumen along with only a small amount of tumour cells (approximately 1% of the cells sloughed). Thus, the DNA of interest represents only a very small percentage of the total DNA isolated from stool. Therefore, along with the exploration of suitable DNA markers, techniques for improved DNA isolation and enrichment of the human DNA component from faecal samples have been developed for more sensitive cancer detection.

The initial DNA isolation techniques typically recovered DNA from 10g to 4g stool and more conveniently purified the human DNA component using streptavidin-bound magnetic beads (Dong et al., 2001; Ahlquist et al., 2000). Further improvements in recovery of target human DNA from stool comprised an electrophoresis-driven separation of target DNA sequences, using oligonucleotide capture probes immobilized in an acrylamide gel (Whitney et al., 2004). Later, when DNA integrity proved to be a suitable marker it was also important to prevent degradation during sample handling. Improved results were obtained with stool samples frozen as quickly as possible after collection. Alternatively,

stabilization buffer was added to the stool samples before further transport (Olson et al., 2005). A recent improvement involves the use of an MBD column to extract methylated human DNA in a high background of fecal bacterial DNA (Zou et al., 2007). However, despite these advances, current tools for cancer detection in faecal samples are still unsatisfactory.

Cancer at its early stage may release its cells or free DNA into blood through apoptosis, necrosis or local angiogenesis, which establishes a basis for blood-based cancer testing. The usefulness of DNA methylation markers for detecting colorectal cancers in serum and plasma has been demonstrated (Grady et al., 2001, Leung et al., 2005; Nakayama et al., 2007). However, the potential use of serum and plasma for cancer detection is hampered by the limited level of methylated DNA present in the total DNA collected from plasma and serum samples (Zou et al. (2002) Clin Cancer Res 188-91). A further drawback is the partial degradation of the methylated DNA due to bisulfite treatment, a treatment step required by many techniques that monitor DNA methylation.

Methods and compositions for detection of early colorectal cancer or pre-cancer using blood and body fluids have been described.

WO 2006/113770 describes methods in which samples are pooled and concentrated in an attempt to maximize DNA input per reaction. The initial processing of 45 ml of blood allowed a median DNA recovery of 3.86 ng/ml plasma. This was shown to result in a sensitivity of 57% and specificity of 96% for

detection of colorectal cancer using a specific real-time assay for detecting whether The Septin 9 gene was methylated. Bisulphite treatment was focused on large volume treatment and achieving maximal conversion.

Lofton-Day et al. (AACR general meeting April 2007, Los Angeles, USA) mention improved detection of colorectal cancer, and obtained a 70% sensitivity and 90% specificity, with the same marker (Septin 9). The proposed method utilised four blood draws (40 ml blood), double centrifugation for plasma recovery and required four PCR reactions to be carried out for each sample tested. Three out of the four reactions used input DNA equivalent to 2 ml of plasma per PCR reaction. The fourth reaction used a 1/10 dilution of this input DNA. Thus, repeated assays were required (at least 4) and an algorithm utilised to determine the final result. A sample was deemed positive if either two out of the three reactions with input DNA equivalent to 2 ml of plasma, or the diluted measurement, were positive for the Septin 9 assay. The improved sensitivity by using the diluted samples indicates the presence of inhibitors in the methods, a phenomena also described by Nakayama et al. (2007, Anticancer Res. 27(3B):1459-63).

The processing of smaller amounts of blood have been described as well (US 20070141582, Hong-Zhi Zou et al. , and Satoru Yamaguchi et al.) but all result in low level of methylated modified DNA detection.

Thus, current blood-based screening methods lack sensitivity.

SUMMARY OF THE INVENTION

The invention, as set out in the claims, is based around the finding that NDRG4/2 subfamily genes, undergoe CpG island promoter methylation-associated gene silencing in human cancer cells, in particular colon cancer cells. The hypermethylation of the NDRG family gene, such as NDRG4 and/or NDRG2, in particular in the promoter region leads to its loss of expression. Importantly, the presence of aberrant methylation at the NDRG4/2 subfamily gene promoter has a prognostic value. The epigenetic loss of NDRG4/2 function can be rescued by the use of DNA demethylating agents and thus provides for a method for treatment. These findings underline the significance of the epigenetic silencing of the NDRG4/2 subfamily genes as one key step in cancer development and may have an important clinical impact for the treatment of the patients.

The present invention is also based upon the discovery of specific genes and panels of genes whose methylation status is linked to the incidence of, or predisposition to, gastrointestinal cancers such as colorectal cancer. Use of these genes for detecting gastrointestinal cancers such as colorectal cancer, in particular in the context of appropriate tissue or faecal (stool) samples or of appropriate blood samples (or derivatives thereof) respectively, has been shown to produce highly sensitive and specific results. The invention provides also for a method for isolating increased amount of DNA from faecal samples, which results in improved sensitivity of detection of colorectal cancer in faecal samples.

The invention also provides a method for determining the methylation status of a gene of interest in a blood based sample, which requires only low volumes of blood sample equivalent to generate specific and sensitive results. This is advantageous since it permits smaller blood samples to be obtained from the subject under test.

Accordingly, in a first aspect, the invention provides a method of detecting a predisposition to, or the incidence of, cancer in a sample comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, cancer.

Subsets of genes for all aspects and embodiments of the invention include an NDRG4/NDRG2 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT and TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 respectively. Each subset may be particularly applicable to bodily fluid samples, such as stool and plasma samples as discussed herein.

By "epigenetic change" is meant a modification in the gene caused by an epigenetic mechanism, such as a change in methylation status or histone acetylation for example. Frequently, the epigenetic change will result in an alteration in the levels of expression of the gene which may be detected (at the RNA or protein level as appropriate) as an indication of the epigenetic change. Often the

epigenetic change results in silencing or down regulation of the gene, referred to herein as "epigenetic silencing". The most frequently investigated epigenetic change in the methods of the invention involves determining the methylation status of the gene, where an increased level of methylation is typically associated with the relevant cancer (since it may cause down regulation of gene expression).

In a related aspect, the invention provides a method of diagnosing cancer or predisposition to cancer comprising detecting epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the gene is indicative for cancer or predisposition to cancer.

The NDRG family genes have been characterised in the art (see, for example, Qu X *et al., 2002* and references cited therein) and their epigenetic silencing can be assessed in terms of DNA methylation status or expression levels as determined by their methylation status.

In one embodiment, the invention provides for a method of diagnosing cancer or predisposition to cancer comprising detecting epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the NDRG2/NDRG4-family gene is detected by determination of the methylation status of the NDRG4/2 family gene and wherein methylation of the gene is indicative for cancer or predisposition to cancer.

Since methylation of the NDRG4/NDRG2 subfamily gene manifests itself in reduced expression of the gene the invention also provides for a method of diagnosing cancer or predisposition to cancer comprising detecting epigenetic

silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the NDRG2/NDRG4-family gene is determined by measurement of expression levels of the gene, wherein reduced expression of the gene is indicative for cancer or predisposition to cancer.

In a related aspect, the invention provides method of prognosis to cancer or predisposition to cancer comprising detecting epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the gene is indicative for cancer development or predisposition to cancer. Preferably, epigenetic silencing is detected by determination of the methylation status and/or measurement of expression levels of the NDRG2/NDRG4-family gene.

The invention also provides a method of detecting a predisposition to, or the incidence of, cancer and in particular a gastrointestinal cancer such as colorectal cancer in a sample comprising detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, and MGMT, and/or TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, cancer and in particular a gastrointestinal cancer such as colorectal cancer. These subsets of genes may be particularly useful where faecal test samples are utilised (and plasma in certain embodiments).

In a related aspect, the invention also provides a method of detecting a predisposition to, or the incidence of, cancer and in particular a gastrointestinal cancer such as

colorectal cancer in a sample and in particular in a blood sample, or derivative thereof comprising detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3(together with any suitable subset or panel thereof), wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, cancer and in particular a gastrointestinal cancer such as colorectal cancer.

By "NDRG2/NDRG4 subfamily gene" is meant any gene which is taken from the subfamily to which NDRG4 and NDRG2 belong and includes according to all aspects of the invention NDRG2 and NDRG4. Note that "NDRG1, NDRG2, NDRG3 and NDRG4" is the standard nomenclature approved by the human genome organisation for the NDRG family genes, to ensure that each symbol is unique. The listed accession number for these genes can be found at www.gene.ucl.ac.uk/nomenclature.

NDRG family genes encompass not only the particular sequences found in the publicly available database entries, but also encompass transcript variants of these sequences. Variant forms of the encoded proteins may comprise post-translational modification, may result from spliced messages, etc…. NDRG4 has transcript variants having the accession numbers NM_020465 and NM_022910. NDRG2 has several transcript variants having the accession numbers , NM_201535, NM_201536, NM_201537, NM_201538, NM_201539, NM_201540, NM_2015401 and NM_016250. 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% identity to sequences in the database entries or sequence listing. Computer programs for determining percent identity are available in the art, including Basic Local Alignment Search Tool (BLAST5) available from the National Center for Biotechnology Information.

GATA4 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 8 (location p23.1-p22) and the gene sequence is listed under the accession numbers AK097060, NM_002052 and ENSG00000136574. The gene encodes GATA binding protein 4.

OSMR is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 5 (location p13.2) and the gene sequence is listed under the accession numbers U60805, NM_003999 and ENSG00000145623. The gene encodes oncostatin M receptor.

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 numbers AB044947 and ENSG00000103034. The gene encodes NDRG family member 4.

GATA5 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 20 and the gene sequence is listed under the accession number ENSG00000130700. The gene encodes GATA binding protein 5.

SFRP1 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 8

(location p11.21) and the gene sequence is listed under the accession numbers AF017987, NM_003012 and ENSG00000104332. The gene encodes secreted frizzled-related protein 1.

ADAM23 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 2 (location q33) and the gene sequence is listed under the accession numbers AB009672 and ENSG00000114948. The gene encodes ADAM metallopeptidase domain 23.

JPH3 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 16 (location q24.3) and the gene sequence is listed under the accession numbers AB042636 and ENSG00000154118. The gene encodes junctophilin 3.

SFRP2 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 4 (location q31.3) and the gene sequence is listed under the accession numbers AF017986 and ENSG00000145423. The gene encodes secreted frizzled-related protein 2.

APC is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 5 (location q21-q22) and the gene sequence is listed under the accession numbers M74088 and ENSG00000134982. The gene encodes adenomatosis polyposis coli.

The MGMT gene encodes O6-methylguanine-DNA methyltransferase (MGMT), which is a cellular DNA repair protein that rapidly reverses alkylation (e.g. methylation) at the O6 position of guanine, thereby neutralizing the cytotoxic effects of

alkylating agents such as temozolomide (TMZ) and carmustine (1-3). MGMT is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 10 (location 10q26) and the gene sequence is listed under the accession numbers M29971, NM_002412 and ENSG00000170430.

BNIP3 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 10 (location 10q26.3) and the gene sequence is listed under the accession numbers U15174 and ENSG00000176171. The gene encodes the BCL2/adenovirus E1B 19kDa interacting protein 3.

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 numbers U89995 and ENSG00000178919. The gene encodes the forkhead box E1 (thyroid transcription factor 2)

JAM3 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 11 (location 11q25) and the gene sequence is listed under the accession numbers AF356518, NM_032801 and ENSG00000166086. The gene encodes the junctional adhesion molecule 3.

PHACTR3 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 20 (location 20q13.32) and the gene sequence is listed under the accession numbers AJ311122, NM_080672 and ENSG00000087495. The gene encodes the phosphatase and actin regulator 3.

TFPI2 is the gene symbol approved by the HUGO Gene Nomenclature Committee. The gene is located on chromosome 7 (location 7q22) and the gene sequence is listed under the accession numbers L27624 and ENSG00000105825. The gene encodes the 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 numbers AB073988 and ENSG00000164736. The gene encodes the 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 6q25) and the gene sequence is listed under the accession numbers AB018339 and ENSG00000131018. The gene encodes the spectrin repeat containing, nuclear envelope 1.

Of course, as appropriate, the skilled person would appreciate that functionally relevant variants of each of the gene sequences may also be detected according to the methods of the invention. For example, the methylation status of a number of splice variants may be determined according to the methods of the invention. Variant sequences preferably 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 *ex vivo* or *in vitro* methods carried out on a test sample, in particular on an isolated test sample. The methods can be used to diagnose any suitable type of cancer. The cancer comprises, consists essentially of or consists of a neoplasia of the gastrointestinal tract such as gastrointestinal cancer in one embodiment. In specific embodiments, the methods of the invention are applied to colorectal cancer, gastric cancer and/or oesophageal cancer. In more specific embodiments, the methods are used to diagnose colorectal cancer, and more particularly to diagnose hereditary nonpolyposis colon cancer and/or sporadic colorectal cancer. Alternatively, the methods are aimed at diagnosis of gastric cancer. Preferably, the methods are used to diagnose colorectal cancer and/or gastric cancer. The methods may be used to detect carcinoma or adenoma, in particular advanced adenoma. The methods may be employed in the diagnosis of both diffuse type and intestinal type carcinomas of the stomach, particularly when the methylation status of NDRG4 is determined. In one embodiment the methods may also include the step of obtaining the sample.

In one specific embodiment, the methods are used to diagnose oesophageal adenocarcinoma. In particular, the methylation status of the NDRG4 gene (promoter) has been shown for the first time herein to be linked with high sensitivity and specificity to the incidence of this particular cancer type. Oesophageal adenocarcinoma may be distinguished from oesophageal squamous cell carcinomas on this basis.

The "test sample" can be any tissue sample or body fluid. Preferably, the test sample is obtained from a human subject. In specific embodiments, the sample is taken from the gastrointestinal tract. The sample may be a colorectal tissue sample or a colon, rectal, oesophageal, stomach or appendix tissue sample or a faecal or blood based sample from a subject. For faecal samples the methods are preferably used with respect to detecting gastrointestinal cancers such as colorectal cancer as discussed herein, but may also be useful in identifying potentially dangerous adenomas. Different markers and panels of markers may be most useful with a specific sample type, such as a tissue, blood based or faecal sample as discussed herein in detail.

Thus, for example, in one embodiment, the methods of the invention involve detecting an epigenetic change, and in particular determining the methylation status, of (at least) the NDRG4 gene in a faecal test sample, wherein detection of the epigenetic change, in particular (hyper)methylation of the NDRG4 gene (promoter) is indicative of gastrointestinal neoplasias/cancer, in particular colorectal cancer, such as adenomas and carcinomas, gastric cancer and other adenocarcinomas of the gastrointestinal tract (such as oesophageal adenocarcinoma) and/or diffuse type and intestinal type carcinomas of the stomach.

The subject may be suspected of being tumorigenic. More specifically the subject may be suspected of suffering from a cancer, such as a gastrointestinal cancer and in particular colorectal cancer, as discussed herein. However, any other suitable test samples in which epigenetic

silencing of the appropriate gene or genes of the invention, for example an NDRG4/NDRG2 subfamily gene, can be determined to indicate the presence of cancer are included within the scope of the invention. Preferred panels and subsets of genes are presented herein which provide sensitive and specific diagnosis, including early stage detection, of a gastrointestinal cancer such as colorectal cancer based upon appropriate samples such as tissue, faecal and plasma samples as discussed herein. Thus, in embodiments in which tissue samples are utilised, the methods of the invention may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, a gastrointestinal cancer such as colorectal cancer. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample for example.

Other DNA-containing sample which may be used in the methods of the invention include samples for diagnostic, prognostic, or personalised medicinal uses. These samples may be obtained from surgical samples, such as biopsies or fine needle aspirates, from paraffin embedded tissues, from frozen tumour tissue samples, from fresh tumour tissue samples or from a fresh or frozen body fluid, for example. Non-limiting examples include whole blood or parts/fractions thereof, bone marrow, cerebrospinal fluid, peritoneal fluid, pleural fluid, lymph fluid, serum, plasma, urine, chyle, ejaculate, sputum, nipple aspirate, saliva, swabs specimens, colon wash specimens and brush specimens. The tissues and

body fluids can be collected using any suitable method, many such methods are well known in the art. Assessment of a paraffin-embedded specimen can be performed directly or on a tissue section. Tissue samples are generally taken from the tissue suspected of being tumourigenic.

In a specific embodiment, the test sample is a blood sample. Any blood sample, or derivative thereof may be utilised. The blood sample, or derivative thereof may comprise, consist essentially or whole blood or any suitable DNA containing parts/fractions thereof. In specific embodiments, the blood sample or derivative thereof comprises, consist essentially of or consists of serum or plasma. The blood sample may be collected using any suitable method, many such methods are well known in the art. In one embodiment, the methods of the invention also incorporate the step of obtaining the blood sample. Any appropriate blood sample may be utilised in the methods of the invention, provided it contains sufficient DNA. In a specific embodiment, the volume of the blood sample, or derivative thereof that is utilised in the methods is around 5 to 15 ml, such as 10 ml.

Blood samples, or derivatives thereof, may be stored prior to use in the methods of the invention once obtained. They may be frozen for example at a suitable temperature, such as around -80°C.

It is preferred that the blood sample, or derivative thereof comprises, consists essentially of or consists of a plasma or serum sample. Plasma may be derived from whole blood by any suitable means. In one embodiment, the plasma sample is

obtained by centrifugation of whole blood. Centrifugation may be carried out at any suitable speed and for any suitable period of time and under any suitable conditions as may be determined by one skilled in the art. For example, centrifugation may be carried out at between around 1000 and 3000g. Centrifugation may be carried out for between around 1, 2, 3, 4, or 5 and 10, 11, 12, 13, 14 or 15 minutes for example. Centrifugation may be carried out at low temperatures, such as between around 0 and 5°C, for example 4°C, to maintain integrity of the sample. Multiple centrifugation steps may be employed in order to obtain the plasma sample. In a specific embodiment, two centrifugation steps are employed to obtain the plasma sample.

In embodiments where blood and in particular plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. As shown below, these genes provide sensitive and specific methods for diagnosing colorectal cancer in plasma samples. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23. Additional genes which may be employed in plasma or serum based methods include TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3.

"Diagnosis" is defined herein to include screening for a disease or pre-indication of a disease, identifying a disease or pre-indication of a disease, monitoring the staging and the state and progression of the disease, checking for recurrence of disease following treatment and monitoring the success of a particular treatment. The methods of the invention may also have prognostic value, and

this is included within the definition of the term "diagnosis". The prognostic value of the methods of the invention may be used as a marker of potential susceptibility to a number of gastrointestinal cancers such as 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.

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 one embodiment, purification involves alcohol precipitation of DNA. Preferred alcohols include ethanol and isopropanol. Suitable purification techniques also include salt-based precipitation methods. Thus, in one specific embodiment 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 an alternative embodiment, the DNA purification technique is based upon use of organic solvents to extract contaminants from cell lysates. Thus, in one embodiment,

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 or blood based 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 in particular to faecal samples include the Wizard Genomic DNA Purification Kit (Promega), the NucliSENS® easyMAG™ (Biomerieux) and nucleic acid purification kits manufactured by Macherey Nagel.

In specific embodiments, where the test sample is a blood based sample, the DNA may be isolated by phenol-chloroform extraction since this has been shown to provide particularly high levels of DNA recovery from the sample.

Where blood based test samples are employed, the ChargeSwitch procedure may be utilised for example.

Suitable methods and kits for isolating DNA from blood samples are commercially available. Examples, each of which may be utilised in the methods of the invention are provided in the table below.

Table 1: Kits and methods for isolating DNA from blood samples.

Kit	Company	Method
UltraClean-htp™ BloodSpin™ DNA	Mo Bio Laboratories, Inc.	Silica-membrane
PAXgene Blood DNA Kit	Qiagen	isopropanol
QIAamp DNA Blood Maxi/Mini Kit	Qiagen	Silica-membrane
FlexiGene DNA Kit	Qiagen	isopropanol
GeneCatcher gDNA 3-10 ml Blood	Invitrogen	magnetic beads
BC-204-10ml-blood - Blood 10 ml	Baseclear	magnetic beads
ZR Genomic DNA I Kit	Zymo research	magnetic beads
DNAzol BD	MRC, Inc.	isopropanol
Gentra pureGene* DNA Purification Blood	Fischer	isopropanol
MasterPure Whole Blood DNA	Epicentre Biotech.	isopropanol
Invisorb® Blood Giga Kit	Westburg	isopropanol
100436-10 (Maxi)	Bioron	Silica-membrane
MagNA Pure LC DNA Isolation Kit	Roche	magnetic beads
Nuclisens EasyMag	Biomérieux	magnetic beads
chemagic blood kit special	chemagen	magnetic beads

The QIAamp DNA Blood Maxi kit available from Qiagen and the GeneCatcher gDNA kit from Invitrogen both utilise plasma or serum as starting material.

Thus, as can be derived from table 1, DNA isolation may be carried out using silica-membranes, isopropanol or magnetic bead based methods for example.

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 one embodiment, kits such as the Picogreen® dsDNA quantitation kit available from Molecular Probes, Invitrogen may be employed to quantify the DNA.

"Cancer" is defined herein to include neoplasias. Neoplasia refers to abnormal new growth and thus means the same as tumor, which may be benign or malignant. Particular cancer types which are relevant in accordance with the present invention are discussed above and include those selected from neoplasias of the gastrointestinal tract. Specific examples include colorectal cancer, oesophageal cancer, stomach cancer and gastric cancer.

"Colorectal cancer", also called colon cancer or bowel cancer, is defined to include cancerous growths in the colon, rectum and appendix. Specific markers and panels of markers, as described in greater detail herein, may be particularly applicable to certain cancer types.

Other cancer types which may be relevant in specific (but not all) embodiments of the invention include prostate cancer, breast cancer, ovarian cancer and thyroid cancer.

"Epigenetic silencing" is defined herein to include any alteration in the DNA 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.

Epigenetic silencing of, or an epigenetic change such as methylation in, the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may manifest itself before abnormal new growth/cancer is observable. A subject may be undergoing routine screening and may not necessarily be suspected of having a disease such as a colon neoplasia. Detecting epigenetic silencing of the gene or genes in an adenoma of such a subject may indicate that the probable course of the adenoma is development to a carcinoma and thus there is a predisposition to neoplasia. In such cases, preventive treatment may be recommended and involve resection of the advanced adenoma. These methods may advantageously involve detection of methylation of the NDRG4 gene, in particular using primer set 1, as discussed herein.

"Advanced adenoma" refers to an adenoma in which epigenetic silencing of at least one of the gene linked to colorectal cancer is observed, preferably epigenetic silencing such as methylation of the gene or genes of the invention (such as NDRG4 etc.) is detected.

The most preferred epigenetic change in the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) which is detected comprises, consists essentially of or consists of 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 ("5-mCyt") 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.

Diminished gene expression can be assessed in terms of DNA methylation status or in terms of expression levels as determined by the methylation status of the gene. One method to detect epigenetic silencing is to determine that a gene which is expressed in normal cells is less expressed or not expressed in tumor cells. Accordingly, the invention provides for a method of diagnosing cancer or predisposition to cancer comprising detecting epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the NDRG2/NDRG4-family gene is determined by measurement of expression levels of the gene and wherein reduced expression of the gene is indicative for cancer or predisposition to cancer. The invention also provides a method of detecting a

predisposition to, or the incidence of, a cancer in particular a gastrointestinal cancer such as colorectal cancer in a sample comprising detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4 (or another NDRG4/NDRG2 subfamily member), GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and, MGMT and/or at least one gene selected from, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is determined by measuring expression levels of the at least one gene and wherein low level, reduced level or a lack of expression of the at least one gene is indicative of a predisposition to, or the incidence of, cancer and in particular a gastrointestinal cancer such as colorectal cancer.

In embodiments where blood and in particular plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3. These genes are also useful where faecal test samples are employed. TFPI2 may be a particularly useful marker. Specific genes such as genes slected from TPF12, BNIP3, FOXE1, SYNE1 and SOX17 may be most useful when plasma samples are employed. For stool samples genes such as GATA4, OSMR, NORG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT and also genes selected from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 may usefully be employed. As shown below, these genes provide sensitive and specific methods for diagnosing colorectal cancer in plasma samples. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23. Further panels are discussed below. This may be utilised in

order to diagnose early stage colorectal cancer, in particular stage 0 to II colorectal cancer.

In embodiments in which tissue samples are utilised, the method preferably comprises, consists essentially of or consists of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, a gastrointestinal cancer such as colorectal cancer. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample for example.

In specific embodiments, total loss of protein expression of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is observed in the sample in order to conclude a diagnosis of cancer and in particular a gastrointestinal cancer such as colorectal cancer or predisposition to cancer and in particular a gastrointestinal cancer such as colorectal cancer, or to make a decision on the best course of treatment in accordance with the other methods of the invention, as described herein (which description applies here *mutatis mutandis*). However, partial loss of expression of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and

combinations including panels as discussed herein) may also be relevant, due to methylation of the relevant gene or genes.

The decreased level of expression of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may, as necessary, be measured in order to determine if it is statistically significant in the sample. This helps to provide a reliable test for the methods of the invention. Any method for determining whether the expression level of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is significantly reduced may be utilised. Such methods are well known in the art and routinely employed. For example, statistical analyses may be performed. One example involves an analysis of variance test. Typical P values for use in such a method would be P values of < 0.05 or 0.01 or 0.001 when determining whether the relative expression or activity is statistically significant. A change in expression may be deemed significant if there is at least a 10% decrease for example. The test may be made more selective by making the change at least 15%, 20%, 25%, 30%, 35%, 40% or 50%, for example, in order to be considered statistically significant.

In a specific embodiment of the methods of the invention, the decreased level of expression or activity of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is determined with reference to a control sample. This control sample is preferably taken from normal (i.e. non tumorigenic) tissue in the subject, where expression of the corresponding gene or genes is normal. Additionally or alternatively control samples may also be utilised in which there is known to be a lack of expression of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein).

Suitable additional controls may also be included to ensure that the test is working properly, such as measuring levels of expression or activity of a suitable reference gene in both test and control samples. Suitable reference genes for the present invention include beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA genes such as 18S ribosomal RNA and RNA polymerase II gene (Radonic A. et al., Biochem Biophys Res Commun. 2004 Jan 23;313(4):856-62). In specific embodiments, the reference gene is beta-actin.

Expression of a nucleic acid can be measured at the RNA level or at the protein level. Cells in test samples can be lysed and the mRNA levels in the lysates, or in the RNA

purified or semi-purified from the lysates, determined. Alternatively, methods can be used on unlysed tissues or cell suspensions. Suitable methods for determining expression of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) at the RNA level are well known in the art and described herein.

Methods employing nucleic acid probe hybridization to the relevant transcript(s) of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be employed for measuring the presence and/or level of the respective mRNA. Such methods are well known in the art and include use of nucleic acid probe arrays (microarray technology) and Northern blots. Advances in genomic technologies now permit the simultaneous analysis of thousands of genes, although many are based on the same concept of specific probe-target hybridization. Sequencing-based methods are an alternative. These methods started with the use of expressed sequence tags (ESTs), and now include methods based on short tags, such as serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS). Differential display techniques provide yet another means of analyzing gene expression; this family of techniques is based on random amplification of cDNA fragments generated by restriction digestion, and bands that differ between two tissues identify cDNAs of interest.

In certain embodiments, the levels of gene expression of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) are determined using reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is a well known technique in the art which relies upon the enzyme reverse transcriptase to reverse transcribe mRNA to form cDNA, which can then be amplified in a standard PCR reaction. Protocols and kits for carrying out RT-PCR are extremely well known to those of skill in the art and are commercially available.

In one embodiment, primers useful in RT-PCR carried out on the NDRG4 gene are provided. These primers comprise, consist essentially of or consist of the following sequences:

SEQ ID NO: 1 5′- cctgaggagaagccgctg -3′ (forward)
SEQ ID NO: 2 5′- atgtcatgttccttccagtctgt -3′ (reverse)

SEQ ID NO: 3 5′-GGCCTTCTGCATGTAGTGATCCG-3′ (forward)
SEQ ID NO: 4 5′-GGTGATCTCCTGCATGTCCTCG-3′ (reverse)

Variants of these primers are include within the scope of the invention, as defined herein which definition applies mutatis mutandis.

The RT-PCR can be carried out in a non-quantitative manner. End-point RT-PCR measures changes in expression levels using

three different methods: relative, competitive and comparative. These traditional methods are well known in the art. Alternatively, RT-PCR is carried out in a real time and/or in a quantitative manner. Real time quantitative RT-PCR has been thoroughly described in the literature (see Gibson et al for an early example of the technique) and a variety of techniques are possible. Examples include use of hydrolytic probes (Taqman), hairpin probes (Molecular Beacons), FRET probe pairs (LightCycler (Roche)), hairpin probes attached to primers (Scorpion), hairpin primers (Plexor and Amplifluor), DzyNA and oligonucleotide blocker systems. All of these systems are commercially available and well characterised, and may allow multiplexing (that is, the determination of expression of multiple genes in a single sample).

TAQMAN was one of the earliest available real-time PCR techniques and relies upon a probe which binds between the upstream and downstream primer binding sites in a PCR reaction. A TAQMAN probe contains a 5' fluorophore and a 3' quencher moiety. Thus, when bound to its binding site on the DNA the probe does not fluoresce due to the presence of the quencher in close proximity to the fluorophore. During amplification, the 5' - 3' exonuclease activity of a suitable polymerase such as Taq digests the probe if it is bound to the strand being amplified. This digestion of the probe causes displacement of the fluorophore. Release of the fluorophore means that it is no longer in close proximity to the quencher moiety and this therefore allows the fluorophore to fluoresce. The resulting fluorescence may be measured and is in direct proportion to the amount of target

sequence that is being amplified. These probes are sometimes generically referred to as hydrolytic probes.

In the Molecular Beacons system, the probe is again designed to bind between the primer binding sites. However, here the probe is a hairpin shaped probe. The hairpin in the probe when not bound to its target sequence means that a fluorophore attached to one end of the probe and a quencher attached to the other end of the probe are brought into close proximity and therefore internal quenching occurs. Only when the target sequence for the probe is formed during the PCR amplification does the probe unfold and bind to this sequence. The loop portion of the probe acts as the probe itself, while the stem is formed by complimentary arm sequences (to respective ends of which are attached the fluorophore and quencher moiety). When the beacon probe detects its target, it undergoes a conformational change forcing the stem apart and this separates the fluorophore and quencher. This causes the energy transfer to the quencher to be disrupted and therefore restores fluorescence.

During the denaturation step, the Molecular Beacons assume a random-coil configuration and fluoresce. As the temperature is lowered to allow annealing of the primers, stem hybrids form rapidly, preventing fluorescence. However, at the annealing temperature, Molecular Beacons also bind to the amplicons, undergo conformational reorganisation, leading to fluorescence. When the temperature is raised to allow primer extension, the Molecular Beacons dissociate from their targets and do not interfere with polymerisation. A new hybridisation takes place in the annealing step of every

cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplicon.

Scorpions primers are based upon the same principles as Molecular Beacons. However, here, the probe is bound to, and forms an integral part of, an amplification primer. The probe has a blocking group at its 5' end to prevent amplification through the probe sequence. After one round of amplification has been directed by this primer, the target sequence for the probe is produced and to this the probe binds. Thus, the name "scorpion" arises from the fact that the probe as part of an amplification product internally hybridises to its target sequence thus forming a tail type structure. Probe-target binding is kinetically favoured over intrastrand secondary structures. Scorpions primers were first described in the paper "Detection of PCR products using self-probing amplicons and fluorescence" (Nature Biotechnology. 17, p804-807 (1999)) and numerous variants on the basic theme have subsequently been produced.

In similar fashion to Scorpions primers, Amplifluor primers rely upon incorporation of a Molecular Beacon type probe into a primer. Again, the hairpin structure of the probe forms part of an amplification primer itself. However, in contrast to Scorpions type primers, there is no block at the 5' end of the probe in order to prevent it being amplified and forming part of an amplification product. Accordingly, the primer binds to a template strand and directs synthesis of the complementary strand. The primer therefore becomes part of the amplification product in the first round of amplification. When the complimentary strand is synthesised amplification occurs through the hairpin structure. This

separates the fluorophore and quencher molecules, thus leading to generation of florescence as amplification proceeds.

DzyNA primers incorporate the complementary/antisense sequence of a 10-23 nucleotide DNAzyme. During amplification, amplicons are produced that contain active (sense) copies of DNAzymes that cleave a reporter substrate included in the reaction mixture. The accumulation of amplicons during PCR/amplification can be monitored in real time by changes in fluorescence produced by separation of fluorophore and quencher dye molecules incorporated into opposite sides of a DNAzyme cleavage site within the reporter substrate. The DNAzyme and reporter substrate sequences can be generic and hence can be adapted for use with primer sets targeting various genes or transcripts (Todd et al., Clinical Chemistry 46:5, 625-630 (2000)).

The Plexor™ qPCR and qRT-PCR Systems take advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis. One of the PCR primers contains a fluorescent label adjacent to an iso-dC residue at the 5′ terminus. The second PCR primer is unlabeled. The reaction mix includes deoxynucleotides and iso-dGTP modified with the quencher dabcyl. Dabcyl-iso-dGTP is preferentially incorporated at the position complementary to the iso-dC residue. The incorporation of the dabcyl-iso-dGTP at this position results in quenching of the fluorescent dye on the complementary strand and a reduction in fluorescence, which allows quantitation during amplification. For these multiplex reactions, a primer pair with a different fluorophore is used for each target sequence.

Real time quantitative techniques for use in the invention generally produce a fluorescent read-out that can be continuously monitored. Fluorescence signals are generated by dyes that are specific to double stranded DNA, like SYBR Green, or by sequence-specific fluorescently-labeled oligonucleotide primers or probes. Each of the primers or probes can be labelled with a different fluorophore to allow specific detection. These real time quantitative techniques are advantageous because they keep the reaction in a "single tube". This means there is no need for downstream analysis in order to obtain results, leading to more rapidly obtained results. Furthermore, keeping the reaction in a "single tube" environment reduces the risk of cross contamination and allows a quantitative output from the methods of the invention. This may be particularly important in a clinical setting for the present invention.

It should be noted that whilst PCR is a preferred amplification method, to include variants on the basic technique such as nested PCR, equivalents may also be included within the scope of the invention. Examples include without limitation isothermal amplification techniques such as NASBA, 3SR, TMA and triamplification, all of which are well known in the art and commercially available. Other suitable amplification methods without limitation include the ligase chain reaction (LCR) (Barringer et al, 1990), MLPA, selective amplification of target polynucleotide sequences (US Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (US Patent No 4,437,975), invader technology (Third Wave Technologies, Madison, WI), strand displacement technology,

arbitrarily primed polymerase chain reaction (WO90/06995) and nick displacement amplification (WO2004/067726).

Suitable methods for determining expression of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) at the protein level are also well known to one of skill in the art. Examples include western blots, immunohistochemical staining and immunolocalization, immunofluorescence, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation assays, complement fixation assay, agglutination reactions, radioimmunoassay, flow cytometry, mass spectrophotometry, and equilibrium dialysis. These methods generally depend upon a reagent specific for identification of the appropriate gene product. Any suitable reagent may be utilised such as lectins, receptors, nucleic acids, antibodies etc. The reagent is preferably an antibody and may comprise monoclonal or polyclonal antibodies. Fragments and derivatized antibodies may also be utilised, to include without limitation Fab fragments, ScFv, single domain antibodies, nano-antibodies, heavy chain antibodies, aptamers etc. which retain gene product binding function. Any detection method may be employed in accordance with the invention. Proteins may be identified on the basis of charge, polarity, amino acid sequence etc. by a range of methods, including SDS-PAGE and amino acid sequencing for example. The nature of the reagent is not limited except that it must be capable of specifically identifying the appropriate gene product.

Of course, in the case of a positive diagnosis of cancer and in particular gastrointestinal cancer such as colorectal cancer, there will be reduced levels of the relevant protein, and perhaps no protein at all. In one embodiment this will present a negative result, if the protein specific reagent is one which binds to the wild type or full length protein. In this case, use of suitable controls ensures that false diagnoses will not be made, for example caused by degraded or non-specific reagents. Thus, the same reagent can be tested on samples in which it is known that the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is expressed at the protein level. A positive result in this control sample, combined with a negative result in the test sample provides a confident diagnosis of cancer and removes any doubt over the quality of the reagent.

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 one embodiment, 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 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.

As discussed in the experimental section, epigenetic silencing resulting in diminished expression of the NDRG4/NDRG2 subfamily gene has been shown in a range of gastrointestinal cancers such as colorectal cancer and gastric cancer. Thus, in one embodiment, the invention provides for a method of diagnosing colorectal cancer and/or gastric cancer or another gastrointestinal cancer as defined herein, predisposition to colorectal cancer and/or gastric cancer or another gastrointestinal cancer as defined herein, comprising detecting epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the NDRG2/NDRG4-family gene is determined by measurement of expression levels of the gene and wherein reduced expression of the gene is indicative for colorectal cancer and/or gastric cancer or another gastrointestinal cancer as defined herein, predisposition to colorectal cancer and/or gastric cancer or another gastrointestinal cancer as defined herein, or progression of adenoma to carcinoma. Preferably, the gene is NDRG2, or NDRG4, or a combination of NDRG2 and NDRG4.

As exemplified in the experimental section, epigenetic silencing resulting in diminished expression of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 has been shown to be sensitively and

specifically linked with the incidence of gastrointestinal cancer and in particular colorectal cancer. Thus, in a further embodiment, the invention provides for a method of diagnosing gastrointestinal cancer and in particular colorectal cancer or predisposition to gastrointestinal cancer and in particular colorectal cancer comprising detecting epigenetic silencing of at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein epigenetic silencing of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 is determined by measurement of expression levels of the gene and wherein reduced expression of the gene is indicative for gastrointestinal cancer and in particular colorectal cancer, predisposition to gastrointestinal cancer and in particular colorectal cancer, or progression of adenoma to carcinoma. These markers may usefully be employed when faecal test samples are utilised.

As is also discussed in the experimental section, epigenetic silencing resulting in diminished expression of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT has been shown to be sensitively and specifically linked with the incidence of colorectal cancer in specific tissue and bodily fluid, such as faecal and blood-based samples. Thus, in one specific embodiment, the invention provides for a method of diagnosing gastrointestinal cancer and in particular colorectal cancer or predisposition to gastrointestinal cancer and in particular colorectal cancer comprising detecting epigenetic silencing of at least one gene selected

from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT in a tissue, faecal or a blood (plasma or serum) sample, or derivative thereof, wherein epigenetic silencing of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT is determined by measurement of expression levels of the gene and wherein reduced expression of the gene is indicative for gastrointestinal cancer and in particular colorectal cancer, predisposition to gastrointestinal cancer and in particular colorectal cancer, or progression of adenoma to carcinoma. As discussed above, where plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

In alternative and complementary embodiments, in particular where bodily fluid such as faecal and plasma samples are utilised the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3. Panels may be selected from these genes and the other genes of the invention as desired and as discussed herein. Methylation of these genes in stool and plasma samples has been shown for the first time herein to be linked to colorectal cancer. Particularly useful markers, which give good levels of sensitivity and specificity in both plasma and faecal samples include TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3. TFPI2 may be particularly useful. Certain genes such as those selected from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17 may prove most useful when testing plasma samples. This discussion applies to all aspects of the invention as appropriate.

It is noted that the expression of additional genes may also be determined in order to supplement the methods of the invention. In fact, any gene involved in the establishment of cancer, as defined herein and in particular gastrointestinal cancers such as colorectal cancer, gastric cancer and/or oesophageal cancer, may be utilized in combination with the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in the methods of present invention. In certain embodiments, the expression level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is analysed in combination with at least one other gene involved in the establishment of cancer. In one embodiment, at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is combined with at least two other genes involved in the establishment of cancer. In a further embodiment at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) and at least three, four, five or six other genes involved in the establishment

of cancer are combined. Other genes involved in the establishment of (colorectal) cancer may be selected from the group consisting of CHFR, p16, Vimentin, p14, RASSF1a, RAB32, SEPTIN-9, RASSF2A, TMEFF2, NGFR and SMARCA3.

Since epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) manifests itself in methylation of the gene, the methods of the invention preferably involve detecting gene methylation. Accordingly, the invention provides a method of diagnosing cancer or predisposition to cancer, in particular gastrointestinal cancers such as colorectal cancer comprising detecting epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein epigenetic silencing of the at least one gene is detected by determination of the methylation status of the at least one gene and wherein methylation of the at least one gene is indicative for cancer or predisposition to cancer, as defined above and in particular gastrointestinal cancers such as colorectal cancer.

In embodiments where blood and in particular plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. As shown below, these genes provide sensitive and specific

methods for diagnosing colorectal cancer in plasma samples. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23. This may be utilised in order to diagnose early stage colorectal cancer, in particular stage 0 to II colorectal cancer. Additionally or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample.

In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. In addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3, such as from TFPI2, FOXE1, SYNE1, SOZ17, PHACTR3 and JAM3, in particular TFPI2. Two, three, four, five or six etc. gene panels selected from these genes are also envisaged in the present invention.

CpG dinucleotides susceptible to methylation are typically concentrated in the promoter region, exons and introns of

human genes. Promoter, exon and intron regions can be assessed for methylation. In one embodiment, the methylation status of the promoter region of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is determined. A "promoter" is a region extending typically between approximately 1 Kb, 500 bp or 150 to 300 bp upstream from the transcription start site. Frequently, the CpG island surrounding or positioned around the transcription start site of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is analysed to determine its methylation status. Alternatively, the methylation status of the exon and/or intron regions of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be determined as appropriate.

In one embodiment of the methods of the invention, the methylation status of the promoter region of the NDRG4 gene is analysed. In another embodiment, the methylation status of the promoter region of the NDRG2 gene is analysed. Alternatively, the promoter region of NDRG2 and NDRG4 are analysed simultaneously.

In one embodiment, the region of the NDRG4/NDRG2 subfamily gene comprising, consisting essentially of, or consisting of the nucleotide sequence of NDRG4 as set forth as SEQ ID NO: 524 (FIG. 3a) and/or the nucleotide sequence of NDRG2 as set forth as SEQ ID NO: 525 (FIG. 3b) is analysed in order to determine its methylation status.

Various methylation assay procedures are known in the art, and can be used in conjunction with the present invention. These assays rely onto two distinct approaches: bisulphite conversion based approaches and non-bisulphite based approaches. Non-bisulphite based methods for analysis of DNA methylation rely on the inability of methylation-sensitive enzymes to cleave methylation cytosines in their restriction. The bisulphite conversion relies on treatment of DNA samples with sodium bisulphite which converts unmethylated cytosine to uracil, while methylated cytosines are maintained (Furuichi et al., 1970). This conversion results in a change in the sequence of the original DNA. DNA methylation analysis has been performed successfully with a number of techniques including: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR (McMS-PCR), MLPA with or without bisulfite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulfite conversion-specific methylation-specific PCR (BS-MSP)(Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence differences in PCR products of sodium bisulfite - treated DNA), methylation-sensitive single-nucleotide primer extension conformation(MS-SNuPE), methylation-sensitive single-strand

conformation analysis (MS-SSCA), Melting curve combined bisulfite 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 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. Any of these techniques may be utilised in accordance with the present invention, as appropriate.

Additional methods for the identification of methylated CpG dinucleotides 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). Alternatively, the MBD may be obtained from MBP, MBP2, MBP4 or poly-MBD (Jorgensen et al., 2006). In one method, restriction exonuclease digested genomic DNA is loaded onto expressed His-tagged methyl-CpG binding domain that is immobilized to a solid matrix and used for preparative column chromatography to isolate highly methylated DNA sequences. Such methylated DNA enrichment-step may supplement the methods of the invention. Several other methods for detecting methylated CpG islands are well known in the art and include amongst others methylated-CpG island recovery assay (MIRA). Any of these methods may be employed in the present invention where desired.

In specific embodiments, the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) (or portion thereof, especially the CpG islands, as discussed herein) is determined using methylation specific PCR (MSP), or an equivalent amplification technique. The MSP technique will be familiar to one of skill in the art. 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).

A specific example of the MSP technique is designated real-time quantitative MSP (QMSP), which permits reliable quantification of methylated DNA in real time. These 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, labelled primers and/or labelled probes can be used. They represent a specific application of the well known and commercially available real-time amplification techniques such as hydrolytic probes (TAQMAN®), hairpin probes (MOLECULAR BEACONS®), hairpin primers (AMPLIFLUOR®), hairpin probes integrated into

primers (SCORPION®), oligonucleotide blockers (such as the HeavyMethyl technique) and primers incorporating complementary sequences of DNAzymes (DzyNA®), specific interatction between two modified nucleotides (Plexor™) etc as described in more detail herein. Often, these real-time methods are used with the polymerase chain reaction (PCR). In Heavymethyl, described for example in WO02/072880 the priming is methylation specific, but non-extendable oligonucleotide blockers provide this specificity instead of the primers themselves. The blockers bind to bisulfite-treated DNA in a methylation-specific manner, and their binding sites overlap the primer binding sites. When the blocker is bound, the primer cannot bind and therefore the amplicon is not generated. Heavymethyl can be used in combination with real-time or end point detection in the methods of the invention.

Thus, in specific emboidiments, the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is determined by methylation specific PCR/amplification, preferably real-time methylation specific PCR/amplification. In specific embodiments, the real time PCR/amplification involves use of hairpin primers (Amplifluor)/hairpin probes (Molecular Beacons)/hydrolytic probes (Taqman)/FRET probe pairs (Lightcycler)/primers incorporating a hairpin probe (Scorpion)/primers incorporating complementary sequences of DNAzymes that cleave a reporter substrate included in the reaction mixture (DzyNA®)/fluorescent dyes (SYBR Green

etc.)/oligonucleotide blockers/the specific interaction between two modified nucleotides (Plexor). Primers and/or probes can be used to investigate the methylation status of the at least one gene.

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 principally as a tool to obtain convenient results presentation and storage, and at the same time to avoid post-PCR handling. Analyses can be performed only to know if the target DNA is present in the sample or not. 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. In the majority of such cases, the quantification of DNA template is not very important. Amplification products may simply be run on a suitable gel, such as an agarose gel, to determine if the expected sized products are present. This may involve use of ethidium bromide staining and visualisation of the DNA bands under a UV illuminator for example. Alternatively, fluorescence or energy transfer can be measured to determine the presence of the methylated DNA. The end-point PCR fluorescence detection technique can use the same approaches as widely used for Real Time PCR: TaqMan assay, Molecular Beacons, Scorpion, Amplifluor etc. 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 reference genes for the present invention include beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA genes such as 18S ribosomal RNA and RNA polymerase II gene (Radonic A. et al., Biochem Biophys Res Commun. 2004 Jan 23;313(4):856-62). In a particularly preferred embodiment, the reference gene is beta-actin.

In one embodiment, each clinical sample is measured in duplicate and for both C_t values (cycles at which the amplification curves crossed the threshold value, set automatically by the relevant software) copy numbers are calculated. The average of both copy numbers (for each gene) is used for the result classification. To quantify the final results for each sample two standard curves are used, one for either the reference gene (β-actin or the non-methylated marker for example) and one for the methylated version of the marker. The results of all clinical samples (when m-Gene was detectable) are expressed as 1000 times the ratio of "copies m-Gene"/"copies reference gene" or "copies m-Gene"/"copies u-Gene+m-Gene" and then classified accordingly (methylated, non-methylated or invalid) (u=unmethylated; m=methylated).

In one embodiment, primers useful in MSP carried out on the promoter region of the NDRG4 gene are provided. These primers comprise, consist essentially of or consist of the following sequences:

TABLE 2

SEQ ID NO.	NDRG4	Primer	Sense primer	SEQ ID NO.	Antisense primer	Annealing temp	Number of PCR cycles
5	Primer set 1	Flank	GGTTYGTTYGGGATTAGTTTTAGG	6	CRAACAACCAAAAACCCCTC	56	35
7	Primer set 1	U	GATTAGTTTTAGGTTTGGTATTGTTTTGT	8	AAAACCAAACTAAAAACAATACACCA	66	25
9	Primer set 1	M	TTTAGGTTCGGTATCGTTTCGC	10	CGAACTAAAAACGATACGCCG	66	25
11	Primer set 2	Flank	ATYGGGGTGTTTTTTAGGTTT	12	ATACCRAACCTAAAACTAATCCC	56	35
13	Primer set 2	U	GGGTGTTTTTTAGGTTTCGCGTCGC	14	CCTAAAACTAATCCCAAACAAACCA	66	30
15	Primer set 2	M	TTTTTTAGGTTTCGCGTCGC	16	AAACTAATCCCGAACGAACCG	66	30

Where "Flank" = Flanking primers

"U" = Unmethylated NDRG4 specific primers

"M" = Methylated NDRG4 specific primers

Primer set 1 is useful in particular applications for predicting the progression of adenomas. Primer set 2 may provide slightly more sensitive results although both primer sets are clearly useful.

In a further embodiment, primers and probes useful in quantitative MSP carried out on the (promoter region of the) NDRG4 gene are provided. These primers and probes comprise, consist essentially of or consist of the following sequences:

SEQ ID NO: 17 5' - GTATTTTAGTCGCGTAGAAGGC - 3' (forward primer)

SEQ ID NO: 18 5' - AATTTAACGAATATAAACGCTCGAC - 3' (reverse primer) and
SEQ ID NO: 19 5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL (molecular beacon probe)

Further characteristics of these primers and probes are summarized in the experimental part.

In a further embodiment, primers useful in MSP carried out on the promoter region of the NDRG2 gene are provided. These primers comprise, consist essentially of or consist of the following sequences:

Flanking primers:
SEQ ID NO: 20 5′-YGTTTTTTATTTATAGYGGTTTTT-3′ (flank up)
SEQ ID NO: 21 5′- TCCTAATACCTCTCCTCTCTTTACTAC -3′ (flank down)

Unmethylated NDRG2 specific primers:
SEQ ID NO: 22 5′- TTTTATTTATAGTGGTTTTTTGTATTTTTT -3′ (sense)
SEQ ID NO: 23 5′- TCTCCTCTCTTTACTACATCCCAACA -3′(antisense)

Methylated NDRG2 specific primers:
SEQ ID NO: 24 5′- TTTATAGCGGTTTTTCGTATTTTTC -3′ (sense)
SEQ ID NO: 25 5′- CCTCTCTTTACTACGTCCCGACG -3′ (antisense).

In one embodiment, primers and/or probes useful in determining the methylation status of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5 ,SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT (carried out on the promoter region of at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT) are

provided. These primers and/or probes comprise, consist essentially of or consist of the following sequences:

Table 3: Primer sequences and beacon (probe) sequences

SEQ ID NO.			
26	ß-Actin	forward primer	5' - TAGGGAGTATATAGGTTGGGGAAGTT - 3'
27		reverse primer	5' - AACACACAATAACAAACACAAATTCAC - 3'
28		beacon	5'-FAM-CGACTGCGTGTGGGGTGGTGATGGAGGAGGTTTAGGCAGTCG-3'-DABCYL
29	GATA4	forward primer	5' - AGGTTAGTTAGCGTTTTAGGGTC - 3'
30		reverse primer	5' - ACGACGACGAAACCTCTCG - 3'
31		beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-3'-DABCYL
32	GATA5	forward primer	5' - AGTTCGTTTTTAGGTTAGTTTTCGGC - 3'
33		reverse primer	5' - CCAATACAACTAAACGAACGAACCG - 3'
34		beacon	5'-FAM-CGACATGCGTAGGGAGGTAGAGGGTTCGGGATTCGTAGCATGTCG-3'-DABCYL
35	SFRP1	forward primer	5' -TGTAGTTTTCGGAGTTAGTGTCGCGC- 3
36		reverse primer	5' -CCTACGATCGAAAACGACGCGAACG- 3'
37		beacon	5'-FAM-CGACATGCTCGGGAGTCGGGGCGTATTTAGTTCGTAGCGGCATGTCG-3'-DABCYL
38	SFRP2	forward primer	5' - GGGTCGGAGTTTTTCGGAGTTGCGC - 3'
39		reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'
40		beacon	5'-FAM-CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTCGGGCATGTCG -3'-DABCYL
17	NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'
18		reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 3'
19		beacon	5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL
41	APC	forward primer	5'-GAACCAAAACGCTCCCCAT-3'
42		reverse primer	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
43		beacon	5' -FAM-CGTCTGCCCCGTCGAAAACCCGCCGATTAACGCAGACG-3'-DABCYL
44	ADAM23	forward primer	5' - GAAGGACGAGAAGTAGGCG - 3'
45		reverse primer	5' - CTAACGAACTACAACCTTACCGA - 3'
46		beacon	5'-FAM-CGACATGCCCCCGACCCGCACGCCGCCCTGCATGTCG-3'-DABCYL
47	OSMR (3)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
48		reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
47	OSMR (4)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
50		reverse primer	5' - AAAAACTTAAAAACCGAAAACTCG - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
51	JPH3	forward primer	5' - TTAGATTTCGTAAACGGTGAAAAC - 3'
52		reverse primer	5' - TCTCCTCCGAAAAACGCTC - 3'
53		beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGCAGACG-3'-DABCYL
54	MGMT	forward primer	5' - TTTCGACGTTCGTAGGTTTTCGC - 3'
55		reverse primer	5' - GCACTCTTCCGAAAACGAAACG - 3'
56		beacon	5'-FAM-CGTCTCGCGTGCGTATCGTTTGCGATTTGGTGAGTGTTTGGGGCGAGACG-3'-DABCYL

In specific embodiments, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers and probes comprising the nucleotide sequences set forth in Table 4 below to determine the methylation status of the at least one gene. The table presents specific primer and probe combinations for certain preferred genes whose methylation status may be determined according to the methods of the invention.

Table 4: Primer sequences and beacon (probe) sequences

SEQ ID NO			
26	ß-Actin	forward primer	5' - TAGGGAGTATATAGGTTGGGGAAGTT - 3'
27		reverse primer	5' - AACACACAATAACAAACACAAATTCAC - 3'
28		beacon	5'-FAM-CGACTGCGTGTGGGGTGGTGATGGAGGAGGTTTAGGCAGTCG-3'-DABCYL
29	GATA4	forward primer	5' - AGGTTAGTTAGCGTTTTAGGGTC - 3'
30		reverse primer	5' - ACGACGACGAAACCTCTCG - 3'
31		beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-3'-DABCYL
32	GATA5	forward primer	5' - AGTTCGTTTTTAGGTTAGTTTTCGGC - 3'
33		reverse primer	5' - CCAATACAACTAAACGAACGAACCG - 3'
34		beacon	5'-FAM-CGACATGCGTAGGGAGGTAGAGGGTTCGGGATTCGTAGCATGTCG-3'-DABCYL
35	SFRP1	forward primer	5' -TGTAGTTTTCGGAGTTAGTGTCGCGC- 3
36		reverse primer	5' -CCTACGATCGAAAACGACGCGAACG- 3'
37		beacon	5'-FAM-CGACATGCTCGGGAGTCGGGGCGTATTTAGTTCGTAGCGGCATGTCG-3'-DABCYL
38	SFRP2	forward primer	5' - GGGTCGGAGTTTTTCGGAGTTGCGC - 3'
39		reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'
40		beacon	5'-FAM-CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTCGGGCATGTCG -3'-DABCYL
17	NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'
18		reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 3'
19		beacon	5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL
41	APC	forward primer	5'-GAACCAAAACGCTCCCCAT-3'
42		reverse primer	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
43		beacon	5' -FAM-CGTCTGCCCCGTCGAAAACCCGCCGATTAACGCAGACG-3'-DABCYL
44	ADAM23	forward	5' - GAAGGACGAGAAGTAGGCG - 3'

		primer	
45		reverse primer	5' - CTAACGAACTACAACCTTACCGA - 3'
46		beacon	5'-FAM-CGACATGCCCCCGACCCGCACGCCGCCCTGCATGTCG-3'-DABCYL
47	OSMR (3)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
48		reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
47	OSMR (4)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
50		reverse primer	5' - AAAAACTTAAAAACCGAAAACTCG - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
51	JPH3	forward primer	5' - TTAGATTTCGTAAACGGTGAAAAC - 3'
52		reverse primer	5' - TCTCCTCCGAAAAACGCTC - 3'
53		beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGCAGACG-3'-DABCYL
54	MGMT	forward primer	5' - TTTCGACGTTCGTAGGTTTTCGC - 3'
55		reverse primer	5' - GCACTCTTCCGAAAACGAAACG - 3'
56		beacon	5'-FAM-CGTCTCGCGTGCGTATCGTTTGCGATTTGGTGAGTGTTTGGGGCGAGACG-3'-DABCYL

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers comprising the nucleotide sequences set forth in Table 5 below to determine the methylation status of NDRG4. The table presents specific primer and probe combinations for determining the methylation status of this gene and the primer pairs and corresponding probe may be selected according to the table.

Table 5 Primer pairs and probes for determining the methylation status of NDRG4, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
NDRG4_1b	112	17	Forward primer	GTATTTTAGTCGCGTAGAAGGC
		18	Reverse primer	AATTTAACGAATATAAACGCTCGAC
		57	Beacon	CGACATGCAGGGATCGCGGTTCGTTCGGGCATGTCG
NDRG4_13830	105	58	Forward primer	GGTATTTTAGTCGCGTAGAAGGC

		59	Reverse primer	GAATATAAACGCTCGACCCGC
		60	Beacon	CGACATGCGCGGTTCGTTCGGGATTAGTTTTAGGTTCGGCATGTCG
NDRG4_2(MvE)	88	9	Forward primer	TTTAGGTTCGGTATCGTTTCGC
		10	Reverse primer	CGAACTAAAAACGATACGCCG
		61	Beacon	CGTACCCGCGTTTATATTCGTTAAATTTACGCGGGTACG
NDRG4_66292	163	62	Forward primer	TAGTCGCGTAGAAGGCGGA
		63	Reverse primer	GACTACAAAAACGAAAACCGAAC
		64	Beacon	CGACATCGGGTACGTTTTCGCGGCGATGTCG
NDRG4_66293	168	58	Forward primer	GGTATTTTAGTCGCGTAGAAGGC
		65	Reverse primer	CTACAAAAACGAAAACCGAAC
		66	Beacon	CGTTTCGCGGGTCGAGCGAAACG
NDRG4_66294	152	62	Forward primer	TAGTCGCGTAGAAGGCGGA
		67	Reverse primer	CGAAAACCGAACTAAAAACGA
		68	Beacon	CGACATGCCGCGGTTCGTTCGGGATTAGTTTTAGGGCATGTCG
NDRG4_66295	90	69	Forward primer	TTTCGTTCGTTTATCGGGT
		70	Reverse primer	CGAACCTAAAACTAATCCCGAAC
		71	Beacon	CGACACGCGTAGAAGGCGGAAGTTACGCGCGTGTCG
NDRG4_66296	160	72	Forward primer	GGTTTCGTAGCGTATTTAGTATAGTTC
		73	Reverse primer	GTAACTTCCGCCTTCTACGC
		74	Beacon	CGACATGCGCGGATCGATCGGGGTGTTTTTTAGGGCATGTCG
NDRG4_66297	143	75	Forward primer	GAGTTGTTTTTGTCGTTTCGTTT
		76	Reverse primer	AACACCTTCATCTCGACGC
		77	Beacon	CGACATGCGGTTCGGTCGAGCGCGCATGTCG
NDRG4_66298	148	78	Forward primer	GTTGTGAGTTGTTTTTGTCGTTTC
		76	Reverse primer	AACACCTTCATCTCGACGC
		79	Beacon	CGACATGCCGTTGTTTCGACGTCGTTATTTAGAGTCGGCATGTCG
NDRG4_66299	144	80	Forward primer	TTTTAGTATTTTTATTTCGGCGTTC
		81	Reverse primer	CTACTCCTACCGCTTCGCTC
		82	Beacon	CGACATCGCGCTCCTCTCCCCGATGTCG
NDRG4_66300	151	83	Forward primer	CGGTGTTTTAGTATTTTTATTTCGG
		84	Reverse primer	AACTACTCCTACCGCTTCGCT
		85	Beacon	CGACATCGGTTTTGGGTGGCGGCGATGTCG
NDRG4_66301	120	80	Forward primer	TTTTAGTATTTTTATTTCGGCGTTC
		86	Reverse primer	CTCTCCTACCGCTCCGCTC
		87	Beacon	CGACATCGCTCCTCTCCCCGACTCGATGTCG
NDRG4_66302	125	83	Forward primer	CGGTGTTTTAGTATTTTTATTTCGG
		86	Reverse primer	CTCTCCTACCGCTCCGCTC
		88	Beacon	CGACATGCCGAACGCGCTACCCCGCATGTCG

NDRG4_66303	95	89	Forward primer	CGAGTCGTTTTAGTTTTCGGT
		90	Reverse primer	TACTCACAAATACCGCCCG
		91	Beacon	CGACATCGGAAAGTGGCGGTCGGTTGCGATGTCG
NDRG4_66304	85	92	Forward primer	TTCGGTGAATTTTAGGAGGC
		93	Reverse primer	TCGAACGACGAACACGAAA
		94	Beacon	CGACATGCGCGGGGTGGGTGCGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Table 6 and 7 below to determine the methylation status of GATA5. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 6 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of GATA5 and Table 7 incorporates Amplifluour sequences which allow the primers to act as hairpin primers, thus facilitating quantitative detection (as discussed in detail herein).

Table 6 - Primer pairs and probes for determining the methylation status of GATA5, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
GATA5_12656	94	95	Forward primer	TTCGGGTTGGAGTATTTATTAGC
		96	Reverse primer	CGAACTTCCAATCTTCGACC
		97	Beacon	CGACATGCGGCGGTGGCGGTGGGTCGGCATGTCG
GATA5_12659	102	98	Forward primer	GATTTTTCGGGGTTTACGAAG
		99	Reverse primer	GAAACTTAACGACAAAAACGCA
		100	Beacon	CGACATGCGTTTAGTTGTATTGGTTCGGGTTTCGCATGTCG
GATA5_12666	107	101	Forward primer	GGTTTGTATTCGGATTCGGTC
		102	Reverse	TCGATAACAACGTCCTACACG

			primer	
		103	Beacon	CGACATGCGAAGGTGGGTTTGCGGTTTGGGAGGTCGCATGTCG
GATA5_12669	111	104	Forward primer	TAGGGTGCGGGTTTGTATTC
		105	Reverse primer	AACAACGTCCTACACGACC
		106	Beacon	CGACATGCGTATTTATCGAAGGTGGGTTTGCGGTTTGCATGTCG
GATA5_66212	118	107	Forward primer	TAGTTGGTGTAGTAGAGGTCGGC
		108	Reverse primer	GACCTAAATCTCGCTTCCGT
		109	Beacon	CGACATGCCGAGGGAGATTGGAGTGAGTTTCGCATGTCG
GATA5_66213	139	110	Forward primer	TATAGCGTGGTGTTGGTCGT
		111	Reverse primer	CTAAATCTCGCTTCCGTCC
		112	Beacon	CGACATGCGCGAGGGAGATTGGAGTGAGTTTCGCATGTCG
GATA5_66215	80	113	Forward primer	GGTGTCGAGGTTTTTAAGGTTTC
		114	Reverse primer	TCACTTTCTAACGAAAACGACT
		115	Beacon	CGACATGCGGGACGGGATGGGTTTTTGCGGGCATGTCG
GATA5_66216	124	116	Forward primer	GTAGTTTCGGAGTTGGGTGTC
		117	Reverse primer	AAAAACGACTCTTCCCGATT
		118	Beacon	CGACATGCGAGGGACGGGATGGGTTTTTGCATGTCG
GATA5_66217	118	116	Forward primer	GTAGTTTCGGAGTTGGGTGTC
		119	Reverse primer	GACTCTTCCCGATTACAACG
		120	Beacon	CGACATGCGAGGGACGGGATGGGTTTTTGGCATGTCG
GATA5_66218	71	121	Forward primer	TTTTGCGTTAAAGGGTCGG
		122	Reverse primer	CGAAACCTTAAAAACCTCGACA
		123	Beacon	CGACATGCCGGGGTTTTAAAGGTAGTTTCGGAGTTGGCATGTCG
GATA5_66219	90	124	Forward primer	GATGTCGTTGCGTTCGTTT
		125	Reverse primer	CCGAAAACCTTAAAAACCTCG
		126	Beacon	CGACATGCCGGCGGGGTTTTAAAGGTAGTTTCGGCATGTCG
GATA5_66220	98	127	Forward primer	GTTTTGCGGATGTCGTTGC
		125	Reverse primer	CCGAAAACCTTAAAAACCTCG
		126	Beacon	CGACATGCCGGCGGGGTTTTAAAGGTAGTTTCGGCATGTCG
GATA5_66221	158	128	Forward primer	TAGGGGTTTTGCGGATGTC
		114	Reverse primer	TCACTTTCTAACGAAAACGACT
		126	Beacon	CGACATGCCGGCGGGGTTTTAAAGGTAGTTTCGGCATGTCG
GATA5_66222	150	129	Forward primer	TCGAGATTGTGGAGTTTTCGT
		130	Reverse primer	TAAAAACCTCGTACTCCGCC
		131	Beacon	CGACATCGGTTTGGGAGGTCGTGTAGGACGATGTCG
GATA5_66223	103	129	Forward primer	TCGAGATTGTGGAGTTTTCGT
		132	Reverse primer	GTAACCCAATCCTAAACTACCGA
		131	Beacon	CGACATCGGTTTGGGAGGTCGTGTAGGACGATGTCG
GATA5_662	112	133		
			Forward	GGTTTGTATTCGGATTCGGT

			primer	
24		134	Reverse primer	ACCCTTCGATAACAACGTCC
		135	Beacon	CGACATGCCGTATTTATCGAAGGTGGGTTTGCGGGCATGTCG
GATA5_662 25	76	136	Forward primer	GTTTCGAGATTGTGGAGTTTTC
		137	Reverse primer	GATAACAACGTCCTACACGACC
		138	Beacon	CGACATGCCGAAGGTGGGTTTGCGGTTTGGGGCATGTCG
GATA5_662 26	163	139	Forward primer	TTATTCGTTTCGTTTCGGG
		140	Reverse primer	AAACCCACCTTCGATAAATACG
		141	Beacon	CGACATCGTTTTTGGTAGGGAGGTTCGGATCGATGTCG
GATA5_662 27	164	142	Forward primer	CGGGGTGTTATTTAGGTTTATTC
		143	Reverse primer	AATACGAAAACTCCACAATCTCG
		144	Beacon	CGACATGCGTTTTTGGTAGGGAGGTTCGGATCGCATGTCG
GATA5_662 28	76	145	Forward primer	CGTTTTTGGTAGGGAGGTTC
		146	Reverse primer	ATCCGAATACAAACCCGCA
		147	Beacon	CGACATGCCGTGGGGGAGGATGAGGGGAGCGTTTCGGCATGTCG
GATA5_662 29	113	142	Forward primer	CGGGGTGTTATTTAGGTTTATTC
		148	Reverse primer	AAACCCGCACCCTACGAAA
		144	Beacon	CGACATGCGTTTTTGGTAGGGAGGTTCGGATCGCATGTCG
GATA5_662 30	161	149	Forward primer	ATTAGTGTAGTTAGACGGGCGG
		150	Reverse primer	GACTCAACCACCAAACACGA
		151	Beacon	CGACATGCGTGGGTTTCGGGGAGTCGCATGTCG
GATA5_662 31	116	95	Forward primer	TTCGGGTTGGAGTATTTATTAGC
		152	Reverse primer	AAACTACGAAACCTCAACGACC
		153	Beacon	CGACATGCGGTGGCGGTGGGTCGCATGTCG
GATA5_662 33	134	154	Forward primer	GTTACGGGAGTTTTGCGTTT
		155	Reverse primer	CGATTCCTCTCCCTCGAAT
		156	Beacon	CGACATGCGAGTTTATGTCGGGTAGGTGTCGCATGTCG
GATA5_662 34	105	157	Forward primer	AATCGTGTTTCGTTCGTATTTTC
		158	Reverse primer	GATATACTCCGAACCCGCC
		159	Beacon	CGACATGCGCGGAGTAGTTTCGTAGGTTGCGGGCATGTCG
GATA5_662 35	121	160	Forward primer	GCGATTTAGGTTAGGGAATCGT
		158	Reverse primer	GATATACTCCGAACCCGCC
		161	Beacon	CGACATGCCGGTGAGGGTTGTATGGAGGCGTCGGCATGTCG
GATA5_662 37	99	162	Forward primer	TTTCGGTGGGGTTTTTAGTC
		163	Reverse primer	GATTCCCTAACCTAAATCGCCT
		164	Beacon	CGACATGCGCGTTAGAAATGCGTGTGGGTAGGAGGCGCATGTCG
GATA5_662 38	72	165	Forward primer	ATTTCGGTGGGGTTTTTAGTC
		166	Reverse primer	CACACGCATTTCTAACGCC

		167	Beacon	CGACATGCCTCTTCCCGAATCCCCGAAAACCGCATGTCG
GATA5_66243	91	168	Forward primer	GGGTTTTATCGTCGCGTGT
		169	Reverse primer	CCGAAAACTAACCTAAAAACGAA
		170	Beacon	CGACATGCCCCGACCCCGCTCACCGGCATGTCG
GATA5_66244	100	171	Forward primer	GGGGTTTACGGGGTTTTATC
		172	Reverse primer	CGAAAACTAACCTAAAAACGAAC
		173	Beacon	CGACATGCGATAATCCCGACCCCGCTCACCGCATGTCG
GATA5_66245	152	174	Forward primer	TTGTTTAGAAATCGAGGAAATCG
		175	Reverse primer	CGACGATAAAACCCCGTAA
		176	Beacon	CGACATGCGAGTTTCGGGTGCGGTTACGCATGTCG
GATA5_66247	163	177	Forward primer	TGTGGTTTCGTTTGTTTAGAAATC
		175	Reverse primer	CGACGATAAAACCCCGTAA
		178	Beacon	CGACATGCGAGTTTCGGGTGCGGTTACGTAACGCATGTCG
GATA5_66250	151	177	Forward primer	TGTGGTTTCGTTTGTTTAGAAATC
		179	Reverse primer	CCCGTAAACCCCCTCGTTA
		180	Beacon	CGACATGCCGCGGGGTTTTCGTTAGTGTATTTCGGCATGTCG
GATA5_66251	85	181	Forward primer	CGTTTGTTTAGAAATCGAGGAAATC
		182	Reverse primer	CATAAAAACGACCGACTCGAA
		183	Beacon	CGACATGCGGGGTTTTCGTTAGTGTATTTCGTTTTAGCATGTCG
GATA5_66252	141	184	Forward primer	TTCGTATTTCGTTATTTATTCGGTT
		185	Reverse primer	GAAACTATAAAACCCCCGCA
		186	Beacon	CGACATGCCGGGTTTTTCGATGGTAGCGTTTTGTACGGCATGTCG
GATA5_66254	131	187	Forward primer	CGAGTTTTCGTTAGGTCGTTT
		188	Reverse primer	ACTCGACTCACACCCGAAC
		189	Beacon	CGACATGCGTACGTTTCGGGCGTCGGTTTTTCGGCATGTCG
GATA5_66255	119	190	Forward primer	CGCGAGTTTTCGTTAGGTC
		191	Reverse primer	CGAACAAATAAAACAACATCGAA
		189	Beacon	CGACATGCGTACGTTTCGGGCGTCGGTTTTTCGGCATGTCG
GATA5_66256	95	192	Forward primer	TCGGGATTTTGGAGGTTTC
		193	Reverse primer	CTACGAATACCGCTACGCC
		194	Beacon	CGACATGCGGGATTTCGTCGGTTTTTTGGCGTAGGGCATGTCG

Table 7 - Additional assay designs: Primer and amplifluor sequences for determining the methylation status of GATA5, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID	Oligonucleotides	5' to 3' Sequences

		NO		
GATA5_12671_S_AMP	90	195	Forward primer	AGCGATGCGTTCGAGCATCGCUTTTTTCGATGTTGTTTTATTTGTTC
		196	Reverse primer	ATAACTATCTACGCCCAACCGA
GATA5_12671_AS_AMP	90	197	Forward primer	TTTTTCGATGTTGTTTTATTTGTTC
		198	Reverse primer	AGCGATGCGTTCGAGCATCGCUATAACTATCTACGCCCAACCGA
GATA5_66214_S_AMP	70	199	Forward primer	AGCGATGCGTTCGAGCATCGCUTTCGTGTAGTTTTATGTAGAGGTCG
		200	Reverse primer	GCTATAACGACGAAACTCGAA
GATA5_66214_AS_AMP	70	201	Forward primer	TTCGTGTAGTTTTATGTAGAGGTCG
		202	Reverse primer	AGCGATGCGTTCGAGCATCGCUGCTATAACGACGAAACTCGAA
GATA5_66236_S_AMP	73	203	Forward primer	AGCGATGCGTTCGAGCATCGCUTTAGGCGTTAGAAATGCGTG
		204	Reverse primer	CACCGAAAATACGAACGAAA
GATA5_66236_AS_AMP	73	205	Forward primer	TTAGGCGTTAGAAATGCGTG
		206	Reverse primer	AGCGATGCGTTCGAGCATCGCUCACCGAAAATACGAACGAAA
GATA5_66239_S_AMP	101	207	Forward primer	AGCGATGCGTTCGAGCATCGCUGGTCGTTAAGTTTGGGTTTATTC
		208	Reverse primer	AAAACTACATAAAAACGCCGCTA
GATA5_66239_AS_AMP	101	209	Forward primer	GGTCGTTAAGTTTGGGTTTATTC
		210	Reverse primer	AGCGATGCGTTCGAGCATCGCUAAAACTACATAAAAACGCCGCTA
GATA5_66240_S_AMP	93	207	Forward primer	AGCGATGCGTTCGAGCATCGCUGGTCGTTAAGTTTGGGTTTATTC
		211	Reverse primer	ATAAAAACGCCGCTACCGC
GATA5_66240_AS_AMP	93	209	Forward primer	GGTCGTTAAGTTTGGGTTTATTC
		212	Reverse primer	AGCGATGCGTTCGAGCATCGCUATAAAAACGCCGCTACCGC
GATA5_66241_S_AMP	78	213	Forward primer	AGCGATGCGTTCGAGCATCGCUCGTTAAGTTTGGGTTTATTCGGT
		214	Reverse primer	CTACCGCGAAACAACTCCG
GATA5_66241_AS_AMP	78	215	Forward primer	CGTTAAGTTTGGGTTTATTCGGT
		216	Reverse primer	AGCGATGCGTTCGAGCATCGCUCTACCGCGAAACAACTCCG
GATA5_66248_S_AMP	86	217	Forward primer	AGCGATGCGTTCGAGCATCGCUGTTTAGAAATCGAGGAAATCGC
		218	Reverse primer	GACTTCCATAAAAACGACCGA
GATA5_66248_AS_AMP	86	219	Forward primer	GTTTAGAAATCGAGGAAATCGC
		220	Reverse primer	AGCGATGCGTTCGAGCATCGCUGACTTCCATAAAAACGACCGA
GATA5_66249_S_AMP	80	217	Forward primer	AGCGATGCGTTCGAGCATCGCUGTTTAGAAATCGAGGAAATCGC
		182	Reverse primer	CATAAAAACGACCGACTCGAA
GATA5_66249_AS_AMP	80	219	Forward primer	GTTTAGAAATCGAGGAAATCGC
		221	Reverse primer	AGCGATGCGTTCGAGCATCGCUCATAAAAACGACCGACTCGAA
GATA5_66257_S_AMP	78	222	Forward primer	AGCGATGCGTTCGAGCATCGCUTTTGCGTGGTCGTAAGGTC

		223	Reverse primer	AAATAAACCCCGAACCGAA
GATA5_66257_AS_AMP	78	224	Forward primer	TTTGCGTGGTCGTAAGGTC
		225	Reverse primer	AGCGATGCGTTCGAGCATCGCUAAATAAACCCCGAACCGAA
GATA5_66246_S_AMP	70	226	Forward primer	AGCGATGCGTTCGAGCATCGCUCGGGGTTTTCGTTAGTGTATTTC
		227	Reverse primer	AAACCGACTTCCATAAAAACGA
GATA5_66246_AS_AMP	70	228	Forward primer	CGGGGTTTTCGTTAGTGTATTTC
		229	Reverse primer	AGCGATGCGTTCGAGCATCGCUAAACCGACTTCCATAAAAACGA

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Tables 8 and 9 below to determine the methylation status of OSMR. The tables present specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 8 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of OSMR and Table 9 incorporates Amplifluour sequences which allow the primers to act as hairpin primers, thus facilitating quantitative detection (as discussed in detail herein).

Table 8 Primer pairs and probes (molecular beacons) for determining the methylation status of OSMR, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
OSMR_1	148	230	Forward primer	GTGTTAAGAGTGCGTAGTAAGACG
		231	Reverse primer	GAAACGAACGTACAAAAACGA
		232	Beacon	CGACATGCCGAAACTATAAATCAACTACGAAACAAACGCGCATGTCG
OSMR_2	142	233	Forward primer	TTAAGTAAACGTTGGGTAGAGGC
		234	Reverse primer	CTCGATAACTTTTCCGACGA

		235	Beacon	CGACATGCCGAGGAGGGGAACGGGTTGTTGGCATGTCG
OSMR_25259	138	236	Forward primer	TGTTCGTTCGTTCGTAAAGTTC
		237	Reverse primer	TACAATTTCCCGTCTTACTACGC
		238	Beacon	CGACATGCGCGGTCGTTTTTTTTCGGGATTGAAGGCATGTCG
OSMR_25260	139	47	Forward primer	TTTGGTCGGGGTAGGAGTAGC
		239	Reverse primer	CACAACCCGAACTTTACGAAC
		240	Beacon	CGACATGCGCGGGGTACGGAGTTTCGGTCGCATGTCG
OSMR_5	130	241	Forward primer	ACGTTGGGTAGAGGCGGTATC
		242	Reverse primer	ATAACTTTTCCGACGAACGAAC
		243	Beacon	CGACATGCACCCATCCCGACTAAACGCGACGCATGTCG
OSMR_66307	120	244	Forward primer	GTATAGTACGGGGTTCGTTCGT
		245	Reverse primer	ACTCGTAAAACCCTTCGCC
		246	Beacon	CGACATGCGGTAGGGCGCGAGTAGAGCGCATGTCG
OSMR_66308	124	247	Forward primer	GGTAGAGGCGGTATCGAGG
		242	Reverse primer	ATAACTTTTCCGACGAACGAAC
		248	Beacon	CGACATGCGGGATGGGTTGCGAAGTTGTCGCATGTCG
OSMR_66309	130	249	Forward primer	ACGTTGGGTAGAGGCGGTA
		242	Reverse primer	ATAACTTTTCCGACGAACGAAC
		250	Beacon	CGACACGCGTTTAGTCGGGATGGGTTGCGTGTCG
OSMR_66310	76	251	Forward primer	CGGTATCGAGGAGGGGAAC
		252	Reverse primer	AAATCCGACAACTTCGCAA
		253	Beacon	CGACATGCGTTGTTGTATTTTCGGTCGCGTTTAGTCGCATGTCG
OSMR_66311	84	247	Forward primer	GGTAGAGGCGGTATCGAGG
		252	Reverse primer	AAATCCGACAACTTCGCAA
		254	Beacon	CGACATGCCGGGTTGTTGTATTTTCGGTCGCGGCATGTCG
OSMR_66312	120	255	Forward primer	TAGGTAGGTAGGTCGGGGGC
		256	Reverse primer	CGAAAATACAACAACCCGTTC
		257	Beacon	CGACATGCGTTGGGTAGAGGCGGTATCGCATGTCG
OSMR_Sid	142	258	Forward primer	TTCGTGCGTTTTTGGTCG
		259	Reverse primer	CGAACTTTACGAACGAACG
		240	Beacon	CGACATGCGCGGGGTACGGAGTTTCGGTCGCATGTCG

Table 9 - Additional assay designs: Primer and amplifluor sequences for determining the methylation status of OSMR, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides	5' to 3' Sequences
OSMR_25258_S_AMP	135	260	Forward primer	AGCGATGCGTTCGAGCATCGCUAGAGTGCGTAGTAAGACGGGA
		261	Reverse primer	ACGTACAAAAACGACCCGAAC
OSMR_25258_AS_AMP	135	262	Forward primer	AGAGTGCGTAGTAAGACGGGA
		263	Reverse primer	AGCGATGCGTTCGAGCATCGCUACGTACAAAAACGACCCGAAC
OSMR_25264_S_AMP	65	264	Forward primer	AGCGATGCGTTCGAGCATCGCUGCGTAGCGTTGTTTTTGTTTC

		265	Reverse primer	CGACTTACCTCTAATTCCGCC
OSMR_25264_AS_AMP	65	266	Forward primer	GCGTAGCGTTGTTTTTGTTTC
		267	Reverse primer	AGCGATGCGTTCGAGCATCGCUCGACTTACCTCTAATTCCGCC
OSMR_66305_S_AMP	142	260	Forward primer	AGCGATGCGTTCGAGCATCGCUAGAGTGCGTAGTAAGACGGGA
		231	Reverse primer	GAAACGAACGTACAAAAACGA
OSMR_66305_AS_AMP	142	262	Forward primer	AGAGTGCGTAGTAAGACGGGA
		268	Reverse primer	AGCGATGCGTTCGAGCATCGCUGAAACGAACGTACAAAAACGA
OSMR_66306_S_AMP	98	260	Forward primer	AGCGATGCGTTCGAGCATCGCUAGAGTGCGTAGTAAGACGGGA
		269	Reverse primer	CTACGAAACAAACGCGAAA
OSMR_66306_AS_AMP	98	262	Forward primer	AGAGTGCGTAGTAAGACGGGA
		270	Reverse primer	AGCGATGCGTTCGAGCATCGCUCTACGAAACAAACGCGAAA
OSMR_66313_S_AMP	71	271	Forward primer	AGCGATGCGTTCGAGCATCGCUCGAGGATTTTTCGAGCGTC
		272	Reverse primer	ATACCGCCTCTACCCAACG
OSMR_66313_AS_AMP	71	273	Forward primer	CGAGGATTTTTCGAGCGTC
		274	Reverse primer	AGCGATGCGTTCGAGCATCGCUATACCGCCTCTACCCAACG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Table 10 below to determine the methylation status of ADAM23. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 6 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of ADAM23.

Table 10 - Primer pairs and probes (molecular beacons) for determining the methylation status of ADAM23, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	oligonucleotides	5' to 3' Sequences (all the beacon sequences are 5'-FAM and 3'-DABCYL)
ADAM23_5	99	275	Forward primer	TAACGTAAAGGGTACGGGG
		276	Reverse primer	GTCCTTCTCCTACTACCTCCGCT
		277	Beacon	CGACATGCCCCGACTCGCCTAACCTCGCAAGCATGTCG
ADAM23_66258	98	278	Forward primer	GTAGTAGTTCGCGGTAGTCGTTT
		279	Reverse primer	AACGCTAACAAACACCGAA
		280	Beacon	CGACATGCGCGGGTTGTAGTTTTGTCGGCGGCATGTCG
ADAM23_66259	169	281	Forward primer	TTCGTAGTCGTTGAAGCGG
		282	Reverse primer	GCGAAACTCGAAACTAAACGA
		283	Beacon	CGACATCGGGAGTGGTTGCGAGGTTAGGCGATGTCG
ADAM23_66260	81	284	Forward primer	GCGTCGTTTTAGTATTTTTAGGTTC
		285	Reverse primer	GACTACTCCCTCCCCCGAC
		286	Beacon	CGACATGCGTTTTCGTAGTCGTTGAAGCGGTCGGCATGTCG
ADAM23_66261	104	287	Forward primer	GTTTTCGCGTCGTTCGTTT
		285	Reverse primer	GACTACTCCCTCCCCCGAC
		288	Beacon	CGACATGCGGTTCGGCGGTAGTTTTCGTAGTCGGCATGTCG
ADAM23_66263	106	289	Forward primer	GGGTACGGGGTTATATTTATCGT
		290	Reverse primer	CTACCGCCTACTTCTCGTCC
		291	Beacon	CGACATCGGGACGAGGCGGCGATGTCG
ADAM23_66264	90	289	Forward primer	GGGTACGGGGTTATATTTATCGT
		276	Reverse primer	GTCCTTCTCCTACTACCTCCGCT
		292	Beacon	CGACATGCCCCCGCGCCTAAAAAACTACTACGGCATGTCG
ADAM23_66265	84	293	Forward primer	GGTACGGGGTTATATTTATCGTTG
		294	Reverse primer	TCTCCTACTACCTCCGCTCG
		295	Beacon	CGACATGCCTCGTCCCGACCCCGCGCATGTCG
ADAM23_66266	125	296	Forward primer	GTCGAGTCGGGGATAAGTTC
		297	Reverse primer	AAAAACTACTACGCCCAACGA
		298	Beacon	CGACATGCGCGGGAAAGTTAACGTAAAGGGTACGCATGTCG
ADAM23_66267	97	296	Forward primer	GTCGAGTCGGGGATAAGTTC
		299	Reverse primer	AACCCCGTACCCTTTACGTT
		300	Beacon	CGACGCGCGTTTTTCGTTTTTTTTTGTAGGGTTTCGCGTCG
ADAM23_66268	133	301	Forward primer	AAGGAAAGGTCGAGTCGGG
		297	Reverse primer	AAAAACTACTACGCCCAACGA
		302	Beacon	CGACATGCGTAGGGTTTCGCGGGAAAGTTAACGGCATGTCG
ADAM23_66269	108	301	Forward primer	AAGGAAAGGTCGAGTCGGG
		303	Reverse primer	TATAACCCCGTACCCTTTACGTT
		304	Beacon	CGACATGCAGTTCGGAGTATACGGATTCGCGCGCATGTCG

ADAM23_66271	97	305	Forward primer	TTCGTCGGTTATACGGAGC
		306	Reverse primer	GACAAAACTACAACCCGCCA
		307	Beacon	CGACATGCGGGAGTTATGAGTTATGAAGTCGTTCGCATGTCG
ADAM23_A	112	308	Forward primer	GAGGTTTTAAGTTGGCGGAGC
		309	Reverse primer	ACTCGAAACTAAACGACGCCC
		277	Beacon	CGACATGCCCCGACTCGCCTAACCTCGCAAGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers comprising the nucleotide sequences set forth in Table 11 below to determine the methylation status of JPH3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table. Table 11 also sets forth specific probes which may be utilised to facilitate (quantitative) detection of the methylation status of JPH3.

Table 11 - Primer pairs and probes (molecular beacons) for determining the methylation status of JPH3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
JPH3_1(MVE)	103	310	Forward primer	TTTAATATGGTGTAGTCGTTAGCGTC
		311	Reverse primer	CCCACCTACGACTACCGCG
		312	Beacon	CGACATGCACGAAACCCGCGAACGACGACGCATGTCG
JPH3_12608	90	313	Forward primer	GGGGTAGGTTTAATTTTGACGAC
		314	Reverse primer	TAAAACCGATACAAACGCCA
		315	Beacon	CGACATGCGGTTGGGAGGACGGTAAGGCGGCATGTCG
JPH3_2	123	316	Forward primer	TGTAGTCGTTAGCGTCGTCGT
		317	Reverse primer	GAAAAACAACTCAAACCCGAA
		318	Beacon	CGACATGCACCCGCGAACGACGACGACGCATGTCG
JPH3_3	88	319	Forward primer	GTAGGTTTAATTTTGACGACGGA
		320	Reverse primer	TTAAAACCGATACAAACGCCA
		321	Beacon	CGACATGCCCGTACGCCTTACCGTCCTCGCATGTCG
JPH3_4	134	322	Forward primer	GATATAGTAGAGTCGCGGTCGTC
		323	Reverse primer	CGATTAACTAAAATTCCTCCGAAA
		324	Beacon	CGACATGCCCGAAAAACGCTCGCGACCCAGCATGTCG

JPH3_5	127	325	Forward primer	GGGGTAGTTTAGGTTCGGGTC
		326	Reverse primer	ATATAATACAACCGCCAACGCC
		327	Beacon	CGACATGCCCGCAACGCGACAACCGCAGCATGTCG
JPH3_67326	122	328	Forward primer	GTAGTCGTTAGCGTCGTCGT
		317	Reverse primer	GAAAAACAACTCAAACCCGAA
		329	Beacon	CGACATGCGCGGTAGTCGTAGGTGGGCATGTCG
JPH3_67329	128	319	Forward primer	GTAGGTTTAATTTTGACGACGGA
		330	Reverse primer	GAAACCGTAACTCCACGAAC
		331	Beacon	CGACATGCGAGGACGGTAAGGCGTACGGGCATGTCG
JPH3_67330	92	319	Forward primer	GTAGGTTTAATTTTGACGACGGA
		332	Reverse primer	ACCCTTAAAACCGATACAAACG
		331	Beacon	CGACATGCGAGGACGGTAAGGCGTACGGGCATGTCG
JPH3_67331	90	313	Forward primer	GGGGTAGGTTTAATTTTGACGAC
		314	Reverse primer	TAAAACCGATACAAACGCCA
		331	Beacon	CGACATGCGAGGACGGTAAGGCGTACGGGCATGTCG
JPH3_67332	115	333	Forward primer	TACGGTTTAATCGGAGGACGTAG
		334	Reverse primer	AACGAAAATAAATACCGCGAA
		335	Beacon	CGACATGCGGGCGCGATCGGAAGTACGGCATGTCG
JPH3_67333	109	333	Forward primer	TACGGTTTAATCGGAGGACGTAG
		336	Reverse primer	AATAAATACCGCGAACCGAA
		335	Beacon	CGACATGCGGGCGCGATCGGAAGTACGGCATGTCG
JPH3_67334	92	333	Forward primer	TACGGTTTAATCGGAGGACGTAG
		337	Reverse primer	GAACCGAACCGAAACGAAA
		335	Beacon	CGACATGCGGGCGCGATCGGAAGTACGGCATGTCG
JPH3_67335	96	51	Forward primer	TTAGATTTCGTAAACGGTGAAAAC
		52	Reverse primer	TCTCCTCCGAAAAACGCTC
		338	Beacon	CGACATGCGCGGTCGTCGGCGGTTTTGGCATGTCG
JPH3_67336	108	339	Forward primer	TGTAATTCGGTTTTAGATTTCGT
		52	Reverse primer	TCTCCTCCGAAAAACGCTC
		338	Beacon	CGACATGCGCGGTCGTCGGCGGTTTTGGCATGTCG
JPH3_67337	91	340	Forward primer	GTTCGTTTTTCGTTTTTCGTTT
		341	Reverse primer	CTAACCTACTAAACCGCGCC
		338	Beacon	CGACATGCGCGGTCGTCGGCGGTTTTGGCATGTCG
JPH3_67338	97	342	Forward primer	GTTTTCGTTCGTTTTTCGTTT
		341	Reverse primer	CTAACCTACTAAACCGCGCC
		338	Beacon	CGACATGCGCGGTCGTCGGCGGTTTTGGCATGTCG
JPH3_67339	120	343	Forward primer	AGTAGTAGTAGTAATGCGGCGGT
		344	Reverse primer	CGAACGAACGAAATACGAAC
		345	Beacon	CGACATGCGCGTTTCGGGTTCGGTTCGGCATGTCG
JPH3_67340	126	346	Forward primer	GGGTAGTTTAGGTTCGGGTC
		326	Reverse primer	ATATAATACAACCGCCAACGCC
		347	Beacon	CGACATGCGCGGGCGTTCGAGGGCGCATGTCG

In specific embodiments, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers and probes comprising the nucleotide sequences

set forth in Table 12 below to determine the methylation status of the at least one gene. The table presents specific primer and probe combinations for certain preferred genes whose methylation status may be determined according to the methods of the invention.

Table 12 - Primer sequences and beacon (probe) sequences

	SEQ ID No		
BNIP3	348	forward primer	5'-TACGCGTAGGTTTTAAGTCGC-3'
	349	reverse primer	5'-TCCCGAACTAAACGAAACCCCG-3'
	350	beacon	5'-FAM-CGACATGCCTACGACCGCGTCGCCCATTAGCATGTCG -3'-DABCYL
FOXE1	351	forward primer	5'-TTTGTTCGTTTTTCGATTGTTC-3'
	352	reverse primer	5'-TAACGCTATAAAACTCCTACCGC-3'
	353	beacon	5'-FAM-CGTCTCGTCGGGGTTCGGGCGTATTTTTTTAGGTAGGCGAGACG-3'-DABCYL
JAM3	354	forward primer	5'-GGGATTATAAGTCGCGTCGC-3'
	355	reverse primer	5'-CGAACGCAAAACCGAAATCG-3'
	356	beacon	5'-FAM-CGACACGATATGGCGTTGAGGCGGTTATCGTGTCG-3'-DABCYL
JPH3	51	forward primer	5'-TTAGATTTCGTAAACGGTGAAAAC-3'
	52	reverse primer	5'-TCTCCTCCGAAAAACGCTC-3'
	53	beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGCAGACG-3'-DABCYL
PHACTR3	357	forward primer	TTATTTTGCGAGCGGTTTC
	358	reverse primer	GAATACTCTAATTCCACGCGACT
	359	beacon	CGACATGCGGGTTCGGTCGGCGCGGGGCATGTCG
TFPI2	360	forward primer	5'-GTTCGTTGGGTAAGGCGTTC-3'
	361	reverse primer	5'-CATAAAACGAACACCCGAACCG-3'
	362	beacon	5'-FAM-CGACATGCACCGCGCACCTCCTCCCGCCAAGCATGTCG-3'-DABCYL
SOX17	363	forward primer	5'-GAGATGTTTCGAGGGTTGC-3'
	364	reverse primer	5'-CCGCAATATCACTAAACCGA-3'
	365	beacon	5'-FAM- CGACATGCGTTCGTGTTTTGGTTTGTCGCGGTTTGGCATGTCG-3'-DABCYL
SYNE1	366	forward primer	5'-GTTGGGTTTTCGTAGTTTTGTAGATCGC-3'
	367	reverse primer	5'-CTACGCCCAAACTCGACG-3'
	368	beacon	5'-FAM-CGACATGCCCCGCCCTATCGCCGAAATCGCATGTCG -3'-DABCYL

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected

from the primers and beacons comprising the nucleotide sequences set forth in Table 13 below to determine the methylation status of BNIP3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 13 - Additional assay designs: Primer and probe sequences for determining the methylation status of BNIP3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
BNIP3_13409	94	369	Forward primer	AGTGTTTAGAGAGTTCGTCGGTT
		370	Reverse primer	CGTAACGAATAAACTACGCGAT
		371	Beacon	CGACATGCGGAGAATTCGGTTTATCGTTCGTCGCGCATGTCG
BNIP3_67227	159	372	Forward primer	TTTTAGGTGGAATTTTAGTTCGC
		373	Reverse primer	CCCTCCTACGAACATACGAAA
		374	Beacon	CGACATGCCGTGCGGTTCGATTCGGGTTTAAGGCATGTCG
BNIP3_67229	160	375	Forward primer	CGGTTTAATTGCGAGACGTAG
		376	Reverse primer	AACGTAAAAACCCCGCGTA
		377	Beacon	CGACATGCCGTGCGGTTCGATTCGGGCATGTCG
BNIP3_67231	107	378	Forward primer	GTTTTCGGGTTTTTGTTCGT
		379	Reverse primer	GACTCTACTCGAACCTCCGCT
		380	Beacon	CGACATGCGGGCGTTCGTTCGTAGGAAGAAGGCATGTCG
BNIP3_67232	141	381	Forward primer	TGAGGACGTGTAGGGAAGC
		382	Reverse primer	AAACGAACAAAAACCCGAAA
		383	Beacon	CGACATGCCGAGCGGTGGGTCGGAGGCATGTCG
BNIP3_67233	153	384	Forward primer	GCGTTAGAGGGTAATTGCG
		385	Reverse primer	CTATAAATTCCTCCGACCGAAC
		386	Beacon	CGACATGCCGCGTCGGGTTGCGGGCATGTCG
BNIP3_67235	94	387	Forward primer	TTTGTATTTCGGGCGTTTC
		388	Reverse primer	GCAACTAAAACACATCCCGC
		389	Beacon	CGACATGCGCGATATGGCGTTAGAGGGTAATTGCGCATGTCG
BNIP3_67236	106	390	Forward primer	GGTTTTTACGGAAGTCGGG
		391	Reverse primer	AATACAAACGCGATATAAAACGAA
		392	Beacon	CGACATGCGCGTTATTTCGTTTCGTGGACGGGCATGTCG
BNIP3_67239	151	393	Forward primer	GATTTCGCGTATTGTTCGG
		394	Reverse primer	GATCCAACTACGAAACGCA
		395	Beacon	CGACATGCGGTTTGGATTCGGGTCGGATCGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 14 below to determine the methylation status of FOXE1. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 14 - Additional assay designs: Primer and probe sequences for determining the methylation status of FOXE1, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
FOXE1_13297	108	396	Forward primer	TTCGTTTCGAGAAGTATTACGC
		397	Reverse primer	GCGCTAAAAACTCAACGTCC
		398	Beacon	CGACATGCGAGTCGTCGGTTAGCGGGTTATTTTCGGCATGTCG
FOXE1_13307	133	399	Forward primer	TTCGTTTTCGGTAGTTATGGC
		400	Reverse primer	GATCCCCTAAACTCTCCGC
		401	Beacon	CGACATGCCGGGTTTTGGATTTTCGCGGTTGTCGGCATGTCG
FOXE1_13317	111	402	Forward primer	CGGAGAGTTTAGGGGATCGT
		403	Reverse primer	CTCTATCTACACCGCGCCA
		404	Beacon	CGACATGCGTTTAGGTTGGTACGCGTTGGAGGGCATGTCG
FOXE1_67265	118	405	Forward primer	ATCGGTGTCGTTTTACGTTTC
		406	Reverse primer	GTAAATCTCCAACCCTACGAAC
		407	Beacon	CGACATGCGCGGAGGGAGGAGTCGGGCATGTCG
FOXE1_67266	125	408	Forward primer	TAGGGAATCGGTGTCGTTTTAC
		409	Reverse primer	CGTAAATCTCCAACCCTACGAAC
		410	Beacon	CGACATGCCGGAGGGAGGAGTCGGTTCGGGCATGTCG
FOXE1_67267	108	411	Forward primer	TGAGGTTTTTCGAGTCGGTT
		412	Reverse primer	CCACAACGTCAAAACGAAA
		413	Beacon	CGACATGCCGGGTTTTAGTCGATCGGGGCATGTCG
FOXE1_67268	100	414	Forward primer	ACGTTCGCGTTATGATTGTC
		415	Reverse primer	CCGACCCCTACTACCGTCT
		416	Beacon	CGACATGCCGTAGTCGGAGGTGTTGGTTATCGGCATGTCG
FOXE1_67270	124	417	Forward primer	GAGGTTATCGTCGTTGTTCGT
		397	Reverse primer	GCGCTAAAAACTCAACGTCC
		418	Beacon	CGACATGCCGCGGGTTGAGTCGTCGGGCATGTCG
FOXE1_67271	116	419	Forward primer	TTAGGGATTATTTTCGGATTTTTC
		420	Reverse primer	TTCTCGAAACGAACAACGAC
		421	Beacon	CGACATGCCGTTCGGTATTAGCGCGTAAGGGGCATGTCG

FOXE1_67274	92	422	Forward primer	CGGTAGAAGGGGAAGCGTT
		423	Reverse primer	CTCATCGCCATAACCATCG
		424	Beacon	CGACATGCGCGTGAGGCGGCGTTCGGCATGTCG
FOXE1_67276	90	351	Forward primer	TTTGTTCGTTTTTCGATTGTTC
		425	Reverse primer	CTATAAAACTCCTACCGCGCC
		426	Beacon	CGACATGCCGGGGTTCGGGCGTATTTTTTTAGGGCATGTCG
FOXE1_67278	98	427	Forward primer	TGTGCGCGTAGAAGAGGTTTC
		428	Reverse primer	CGAAAACAAAACATAAACGACC
		429	Beacon	CGACATGCGGTTAGAGCGAGGGTAGTTAGTATTGGGCATGTCG
FOXE1_67279	90	430	Forward primer	GTGCGCGTAGAAGAGGTTTC
		431	Reverse primer	AAAACATAAACGACCCCCG
		432	Beacon	CGACATGCGAGCGAGGGTAGTTAGTATTGGCGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 15 below to determine the methylation status of JAM3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 15 - Additional assay designs: Primer and probe sequences for determining the methylation status of JAM3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
JAM3_12721	104	433	Forward primer	TGTGTCGGTTTAGAGTATCGTTG
		434	Reverse primer	CAATTACCATAACGACCGCC
		435	Beacon	CGACATGCGTTATTATGGTGTCGGTTCGGTTGGGCATGTCG
JAM3_67314	108	433	Forward primer	TGTGTCGGTTTAGAGTATCGTTG
		436	Reverse primer	GCCCCAATTACCATAACGACC
		435	Beacon	CGACATGCGTTATTATGGTGTCGGTTCGGTTGGGCATGTCG
JAM3_67315	113	437	Forward primer	ATTTATGTGTCGGTTTAGAGTATCG
		436	Reverse primer	GCCCCAATTACCATAACGACC
		435	Beacon	CGACATGCGTTATTATGGTGTCGGTTCGGTTGGGCATGTCG

JAM3_67317	90	438	Forward primer	TCGAGTTTTAGTTTTGGTTGC
		439	Reverse primer	AAATAACGATCCTAACTCCGAAA
		440	Beacon	CGACATGCCGGTTCGGGATTTCGGGAGGCATGTCG
JAM3_67318	133	441	Forward primer	TTTAGTAAGTTTTAGCGTTTACGTC
		442	Reverse primer	GAATAAACTCCTCCCAAACGAA
		443	Beacon	CGACATGCGAGGGTCGTGTTTATCGTTCGGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 16 below to determine the methylation status of PHACTR3. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 16 - Additional assay designs: Primer and probe sequences for determining the methylation status of PHACTR3, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID No	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
PHACTR3_67295	111	444	Forward primer	ATTTAGGTAACGGGTTGGGC
		445	Reverse primer	ACTCCCCGAATACAAACGAA
		446	Beacon	CGACATGCGGTTCGAGGTAGGTGGCGTTGGCATGTCG
PHACTR3_67296	128	447	Forward primer	TTCGTAGAGTGATTTTAGCGTTT
		448	Reverse primer	AACGCCACCTACCTCGAAC
		449	Beacon	CGACATGCGCGGACGTCGGGGAGAATTTAGGGCATGTCG
PHACTR3_67297	92	450	Forward primer	TAATTTGTTTTCGCGTCGG
		451	Reverse primer	CTAAAATCACTCTACGAACGACC
		452	Beacon	CGACATGCGGACGGGAGCGGTTGTTTCGGCATGTCG
PHACTR3_67298	118	453	Forward primer	CGTTTCGGATGTTTTGATTTTAC
		454	Reverse primer	ACTCTACGAACGACCCCGC
		455	Beacon	CGACATGCCGGAGGACGGGAGCGGGCATGTCG
PHACTR3_67299	136	456	Forward primer	TTCGTCGGTGATTTTGGTC

		454	Reverse primer	ACTCTACGAACGACCCCGC
		457	Beacon	CGACATGCCGTCGGTCGGGTTTATGGTCGCATGTCG
PHACTR3_67302	128	458	Forward primer	ACGTTGTTACGAAATCGGG
		459	Reverse primer	AAACGCCTAACTCCAACGAAA
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTTGTCGGCGGCATGTCG
PHACTR3_67303	118	458	Forward primer	ACGTTGTTACGAAATCGGG
		461	Reverse primer	CTCCAACGAAACCTAACGCA
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTTGTCGGCGGCATGTCG
PHACTR3_67304	110	462	Forward primer	CGTTGTTACGAAATCGGGT
		463	Reverse primer	GAAACCTAACGCACCTAAACG
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTTGTCGGCGGCATGTCG
PHACTR3_67305	103	462	Forward primer	CGTTGTTACGAAATCGGGT
		464	Reverse primer	AACGCACCTAAACGCGCTA
		460	Beacon	CGACATGCGGCGTACGTTTTTCGTTTTTTTGTCGGCGGCATGTCG
PHACTR3_67306	93	465	Forward primer	GATACGAGGTAGTCGTTTTCGTT
		358	Reverse primer	GAATACTCTAATTCCACGCGACT
		466	Beacon	CGACATGCGCGGTTATGGGTTCGGTCGGGCATGTCG
PHACTR3_67308	124	467	Forward primer	GACGTTGGGGTTATTTTGC
		358	Reverse primer	GAATACTCTAATTCCACGCGACT
		468	Beacon	CGACATGCGCGATACGAGGTAGTCGTTTTCGTTTTTCGGCATGTCG
PHACTR3_67309	92	469	Forward primer	CGTCGTTTTCGTTTAGTTCGT
		470	Reverse primer	GCAAAATAACCCCAACGTCC
		471	Beacon	CGACATGCGCGGAGGAGGTGGTCGAGGCATGTCG
PHACTR3_67310	133	472	Forward primer	GATTGGGGATAGGAATCGC
		473	Reverse primer	AACGACGAACGAATCGAAA
		471	Beacon	CGACATGCGCGGAGGAGGTGGTCGAGGCATGTCG
PHACTR3_67311	113	472	Forward primer	GATTGGGGATAGGAATCGC
		474	Reverse primer	AACCCGAAACAAATAACGCT
		475	Beacon	CGACATGCGCGGTTTTTCGAATGTAGGCGGGCATGTCG
PHACTR3_67312	101	472	Forward primer	GATTGGGGATAGGAATCGC
		476	Reverse primer	ATAACGCTAAAAACAAAACCCCG
		475	Beacon	CGACATGCGCGGTTTTTCGAATGTAGGCGGGCATGTCG
PHACTR3_67313	92	472	Forward primer	GATTGGGGATAGGAATCGC
		477	Reverse primer	AAAACAAAACCCCGCGAAA
		475	Beacon	CGACATGCGCGGTTTTTCGAATGTAGGCGGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 17 below to determine the methylation status of TFPI2. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 17 - Additional assay designs: Primer and probe sequences for determining the methylation status of TFPI2, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID No	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
TFPI2_12620	117	478	Forward primer	CGGGGTGATAGTTTTCGTG
		479	Reverse primer	CGACTTTCTACTCCAAACGACC
		480	Beacon	CGACATGCGGGTCGGTCGGACGTTCGGCATGTCG
TFPI2_67243	98	481	Forward primer	TAGAAATTGTTGGCGTTGTTTTC
		482	Reverse primer	TACCGAACCCTACTTCTCCGT
		483	Beacon	CGACATGCCGTATAGGAATTGGCGGTAGTTTTGCGTGGCATGTCG
TFPI2_67244	124	484	Forward primer	TAGTCGTCGGCGTAAGGAGC
		485	Reverse primer	AAAACTACGAAAACAACGCCA
		486	Beacon	CGACATGCTGGGTGCGCGTAGGGTAGCATGTCG
TFPI2_67245	120	487	Forward primer	GTGTTCGTTTTATGCGGGG
		488	Reverse primer	TCTTACACAATTTACAACGCGAA
		489	Beacon	CGACATGCCGTTCGGTCGATTTTCGTCGGGCATGTCG
TFPI2_67246	115	490	Forward primer	TTTTTGTTTTAGGCGGTTC
		491	Reverse primer	GACGAAATAACAATCCCCGT
		489	Beacon	CGACATGCCGTTCGGTCGATTTTCGTCGGGCATGTCG
TFPI2_67247	106	492	Forward primer	TTCGTTAGGAAAAGTAGTAGAATCG
		493	Reverse primer	GCCAAACGCTTTCTCGAAC
		494	Beacon	CGACATGCGGGTAAGGCGTTCGAGAAAGCGGCATGTCG
TFPI2_67248	117	478	Forward primer	CGGGGTGATAGTTTTCGTG
		479	Reverse primer	CGACTTTCTACTCCAAACGACC
		495	Beacon	CGACATGCGTCGGTCGGACGTTCGTTTCGGCATGTCG
TFPI2_67250	120	496	Forward primer	GTCGTTAGTTTTTGTACGGGG
		497	Reverse primer	GAAAATCCTAAATACGCGCAA
		498	Beacon	CGACATGCGGGAGGTTTGCGACGATGTTTGTTGGGCATGTCG

In a further specific embodiment, the methods of the invention employ or rely upon or utilise primers selected from the primers and beacons comprising the nucleotide sequences set forth in Table 18 below to determine the methylation status of SOX17. The table presents specific primer combinations for determining the methylation status of this gene and the primer pairs may be selected according to the table.

Table 18 - Additional assay designs: Primer and probe sequences for determining the methylation status of SOX17, with predicted amplification product lengths shown.

Assay name	Amplicon length	SEQ ID NO	Oligonucleotides & probes	5' to 3' Sequences (all the beacons are 5'-FAM and 3'-DABCYL)
SOX17_66067	117	499	Forward primer	GGCGTTAGAGTTTAGTTTCGGT
		500	Reverse primer	TAATCCGAATCCCACGTCC
		501	Beacon	CGACATGCGGTGTAGTTTTGGGCGCGGGCATGTCG
SOX17_66070	131	502	Forward primer	CGGTTTAGTGATATTGCGGG
		503	Reverse primer	ACGTAAAACTCGAACCACGAC
		504	Beacon	CGACATGCGATGTGGTTAATGGAGCGGCGAGGGCATGTCG
SOX17_66071	110	505	Forward primer	TTAGTGATATTGCGGGCGT
		506	Reverse primer	CGACCTAAACGTAAACCTAACGA
		507	Beacon	CGACATGCGGAGCGGCGAGGGCGGCATGTCG
SOX17_66073	92	508	Forward primer	TATTGAGATGTTTCGAGGGTTGC
		509	Reverse primer	CTAAATACGCTATAAACCAAACCG
		510	Beacon	CGACATGCCGGTTCGAAGTCGTCGTTCGTGGCATGTCG
SOX17_66078	96	511	Forward primer	TCGAGTTAAGGGCGAGTTTC
		512	Reverse primer	TCTAAATTCTACTACGCCAACCG
		513	Beacon	CGACATGCGGTGTGGGTTAAGGACGAGCGTAAGGCATGTCG
SOX17_66079	91	511	Forward primer	TCGAGTTAAGGGCGAGTTTC
		514	Reverse primer	ATTCTACTACGCCAACCGCT
		515	Beacon	CGACATGCCGGCGGTCGATGAACGTTTTTATGGGCATGTCG
SOX17_66080	117	516	Forward primer	CGAATAGCGGAGTATCGGTC
		517	Reverse primer	ACTACGCCAACCGCTTACG
		518	Beacon	CGACATCGCGGGTCGAGTTAAGGGCGATGTCG
SOX17_66082	119	519	Forward primer	TTTAGTATTTTGTTTAATTCGGCGT
		520	Reverse primer	AACGAATCCCGTATCCGAC
		521	Beacon	CGACATGCGGATTTTGTTGCGTTAGTCGTTTGCGTTCGCATGTCG

Each and all of these primers and probes form separate aspects of the invention. In particular, the invention relates to primer pairs selected from the primer pairs disclosed herein, including in the tables (which may comprise additional sequence over above the basic sequence listed). Further characteristics of these primers are summarized in the detailed description (experimental part) below. It is noted that variants of these sequences may be utilised in the present invention. In particular, additional sequence specific flanking sequences may be added, for example to improve binding specificity, as required. Variant sequences preferably 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 of the primers and/or probes set forth in any of tables 2, 3, 4, 5 or 6. The primers and probe (including hairpin) structures may incorporate synthetic nucleotide analogues as appropriate or may be RNA or PNA based for example, or mixtures thereof. Similarly alternative fluorescent donor and acceptor moieties/FRET pairs may be utilised as appropriate. In addition to being labelled with the fluorescent donor and acceptor moieties, the primers may include modified oligonucleotides and other appending groups and labels provided that the functionality as a primer in the methods of the invention is not compromised. Similarly alternative fluorescent donor and acceptor moieties/FRET pairs may be utilised as appropriate. Molecules that are commonly used in FRET include fluorescein, 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), rhodamine, 6-carboxyrhodamine (R6G), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-

carboxy-X-rhodamine (ROX), 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL), and 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS). Whether a fluorophore is a donor or an acceptor is defined by its excitation and emission spectra, and the fluorophore with which it is paired. For example, FAM is most efficiently excited by light with a wavelength of 488 nm, and emits light with a spectrum of 500 to 650 nm, and an emission maximum of 525 nm. FAM is a suitable donor fluorophore for use with JOE, TAMRA, and ROX (all of which have their excitation maximum at 514 nm).

Thus, in one embodiment, said donor moiety and said acceptor moiety are selected from 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), rhodamine, 6-carboxyrhodamine (R6G), N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 5-(2'-aminoethyl)aminonapthalene-1-sulfonic acid (EDANS), anthranilamide, coumarin, terbium chelate derivatives, Malachite green, Reactive Red 4, DABCYL, tetramethyl rhodamine, pyrene butyrate, eosine nitrotyrosine, ethidium, and Texas Red. In a further embodiment, said donor moiety is selected from fluorescein, 5-carboxyfluorescein (FAM), rhodamine, 5-(2'-aminoethyl)aminonapthalene-1-sulfonic acid (EDANS), anthranilamide, coumarin, terbium chelate derivatives, Malachite green, and Reactive Red 4, and said acceptor moiety is selected from DABCYL, rhodamine, tetramethyl rhodamine, pyrene butyrate, eosine nitrotyrosine, ethidium, and Texas Red.

In one particular embodiment, said donor moiety is fluorescein or a derivative thereof, and said acceptor

moiety is DABCYL. In specific embodiments, the fluorescein derivative comprises, consists essentially of or consists of 6-carboxy fluorescein.

For all aspects and embodiments of the invention, the primers and in particular the stem loop/hairpin structures, and/or the probes (as appropriate upon the form of detection employed) may be labelled with donor and acceptor moieties during chemical synthesis of the primers or probes or the label may be attached following synthesis using any suitable method. Many such methods are available and well characterised in the art.

It is noted that the specific exemplified probe types (such as the hairpin probe type employed in tables 7 and 9) may be replaced as appropriate with a different probe (or primer) type as appropriate. Equivalents are discussed herein and may be utilised as appropriate.

In a further embodiment, bisulphite sequencing is utilised in order to determine the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). Primers may be designed for use in sequencing through the important CpG islands in the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). Thus,

primers may be designed in both the sense and antisense orientation to direct sequencing across the promoter region of the relevant gene or genes.

In one embodiment, in which the NDRG4 and/or NDRG2 gene is sequenced, bisulphite sequencing may be carried out by using sequencing primers which comprise, consist essentially of or consist of the following sequences, and which may be used in isolation or in combination to sequence both strands:

NDRG4 primers

SEQ ID NO: 570 5'- gatyggggtgttttttaggttt -3' (forward)
wherein "Y" represents a pyrimidine mucleotide

SEQ ID NO: 6 5'- craacaaccaaaaaccccctc -3' (reverse)
Wherein "r" represents a purine nucleotide.

NDRG2 primers

SEQ ID NO: 522 5'- tttgttggttatttttttttttatttttt -3' (forward)

SEQ ID NO: 523 5'- cccccaaactcaataataaaaac -3' (reverse)

These sequencing primers form a further aspect of the invention, with suitable variants being included within the scope of the invention (the discussion of which applies mutatis mutandis here).

Other nucleic acid amplification techniques, in addition to PCR (which includes real-time versions thereof and variants such as nested PCR), may also be utilised, as appropriate, to detect the methylation status of the at least one gene

selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). 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 technlology, 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.

Sequence variation that reflects the methylation status at CpG dinucleotides in the original genomic DNA offers two approaches to primer design. Both primer types may be utilised in the methods of the invention either alone or in combination. 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. Such primers are used in bisulphite genomic sequencing, COBRA and Ms-SnuPE for example. Secondly, primers may be designed

that anneal specifically with either the methylated or unmethylated version of the converted sequence. 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.

One way to distinguish between modified and unmodified DNA is to hybridize oligonucleotide primers which specifically bind to one form or the other of the DNA. After hybridization, an amplification reaction can be performed and amplification products assayed. 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.

Another way to distinguish between modified and unmodified DNA is to use oligonucleotide probes which may also be specific for certain products. Such probes may be hybridized directly to modified DNA or to amplification products of modified DNA. Oligonucleotide probes can 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 technique, amplification is achieved with the use of primers specific for the sequence of the gene whose methylation status is to be assessed. In order to provide specificity for the nucleic acid molecules, primer binding sites corresponding to a suitable region of the sequence may be selected. The skilled reader will appreciate that the nucleic acid molecules may also include sequences other than primer binding sites which are required for detection of the methylation status of the gene, for example RNA Polymerase binding sites or promoter sequences may be required for isothermal amplification technologies, such as NASBA, 3SR and TMA.

TMA (Gen-probe Inc.) is an RNA transcription amplification system using two enzymes to drive the reaction, namely RNA polymerase and reverse transcriptase. The TMA reaction is isothermal and can amplify either DNA or RNA to produce RNA amplified end products. TMA may be combined with Gen-probe's Hybridization Protection Assay (HPA) detection technique to allow detection of products in a single tube. Such single tube detection is a preferred method for carrying out the invention.

Whilst the genes (in particular promoters) of the invention appear to be unmethylated in normal tissues, and thus the detection of methylation (or indeed a lack of methylation) in these genes is readily observable as being significant in terms of a cancer diagnosis and also in selecting suitable treatment regimens and for determining the likelihood of successful treatment or resistance to treatment with certain anti-cancer agents etc, when determining methylation status,

it may be beneficial to include suitable controls in order to ensure the method chosen to assess this parameter is working correctly and reliably. For example, 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 (i.e. a conclusion of a lack of methylation is made even though the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may, in fact, be methylated). 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 SssI methyltransferase. In one specific embodiment, the NDRG4/NDRG2 subfamily gene, preferably the NDRG4 and/or NDRG2 gene, may be assessed in normal lymphocytes, following treatment with SssI methyltransferase, as a positive control.

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 carrying out an amplification in the absence of DNA (for example by using a water only sample). The former experiment acts as a negative control to ensure that false positive results are not obtained (i.e. a conclusion of methylation is made even though the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5,

SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein, such as at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC in one specific embodiment) may, in fact, be unmethylated). The gene may be one which is known to be unmethylated in the sample under investigation or it may have been artificially demethylated, for example by using a suitable DNA methyltransferase inhibitor, such as those discussed in more detail below. In one specific embodiment, the NDRG4/NDRG2 subfamily gene, in particular the NDRG4 and/or NDRG2 gene, may be assessed in normal lymphocytes as a negative control, since it has been shown for the first time herein that the NDRG4 and/or NDRG2 gene is unmethylated in normal tissues.

The application of the methods of present invention to extremely small amounts of abnormally-methylated DNA, that are released into collected fluids, in particular stools, may require the generation and amplification of a DNA library before testing for methylation of any specific gene. Suitable methods on whole genome amplification and libraries generation for such amplification (e.g. Methylplex and Enzyplex technology, Rubicon Genomics) are described in US2003/0143599, WO2004/081225 and WO2004/081183 for example. In addition, WO2005/090507 describes library generation/amplification methods that require either bisulfite conversion or non-bisulfite based application. Bisulfite treatment may occur before or after library construction and may require the use of adaptors resistant to bisulfite conversion. Meth-DOP-PCR (Di Vinci et al, 2006), a modified degenerate oligonucleotide-primed PCR

amplification (DOP-PCR) that is combined with MSP, provides another suitable method for specific detection of methylation in small amounts of DNA. Improved management of patient care may require these existing methods and techniques to supplement the methods of the invention.

As discussed in the experimental section, epigenetic silencing resulting in methylation of the NDRG4/NDRG2 subfamily gene has been shown in a number of gastrointestinal cancers such as colorectal cancer and/or gastric cancer, stomach and oesophageal cancers, in particular oesophageal carcinomas. Thus, in specific embodiments, the invention provides for a method of diagnosing a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or predisposition to a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer comprising detecting the methylation status of the NDRG4/NDRG2 subfamily gene, wherein methylation of the gene is indicative for a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer, or predisposition to a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer. Preferably, the gene is NDRG2, or NDRG4, or a combination of NDRG2 and NDRG4.

Whilst the epigenetic change, in particular methylation status, of any of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed

herein, such as at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT) may be determined in order to diagnose a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto. In specific embodiments, the at least one gene may be selected from GATA4, OSMR, NDRG4 and SFRP2, in particular where faecal samples are utilized. Detecting an epigenetic change, in particular methylation, in these genes results in a particularly sensitive and specific diagnostic method. In a further embodiment, where plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and particularly selected from OSMR, NDRG4, GATA5 and ADAM23, in particular where plasma or serum samples are utilised. Detecting an epigenetic change, in particular methylation, in these genes results in a particularly sensitive and specific diagnostic method.

Additionally or anternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer. The tissue sample may comprise, consist essentially of or consist of a

colon and/or rectal and/or appendix sample for example, as discussed herein above.

In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. in addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3, such as from TFPI2, FOXE1, SYNE1, SOZ17, PHACTR3 and JAM3, in particular TFPI2.

Moreover, in order to improve the sensitivity of the methods of the invention the methods may comprise detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, cancer and in particular gastrointestinal cancers as defined herein, such as colorectal cancer. The panel of genes may comprise/consist essentially of or consist of two, three, four, five or six genes.

Certain panels of genes have been found to result in particularly sensitive methods for detecting a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto - especially colorectal cancer. Accordingly, in one embodiment, the panel of genes comprises, consists essentially of or consists of GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2, NDRG4 and SFRP2, APC and SFRP2, APC and OSMR, APC and GATA4,

APC and NDRG4, MGMT and OSMR, MGMT and GATA4, MGMT and NDRG4, MGMT and SFRP2, MGMT and APC, SFRP1 and MGMT, SFRP1 and OSMR, SFRP1 and GATA4, SFRP1 and NDRG4, SFRP1 and SFRP2, SFRP1 and APC, GATA5 and SFRP1, GATA5 and MGMT, GATA5 and OSMR, GATA5 and GATA4, GATA5 and NDRG4, GATA5 and SFRP2 or GATA5 and APC. Suitable panels incorporating other genes such as ADAM23 and/or JPH3 are also envisaged in the present invention. These embodiments are of particular applications to faecal test samples.

Further useful panels of genes comprise, consist essentially of or consists of SFRP1, SFRP2 and APC or SFRP2, OSMR and APC. Further panels of genes comprise, consist essentially of or consist of GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2, GATA4, NDRG4 and SFRP2 or OSMR, NDRG4 and SFRP2. One specifc four gene panel consists of GATA4, OSMR, NDRG4 and SFRP2. One specific panel of at least six genes comprises, consists essentially of or consists of NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT. These panels may usefully be applied to faecal test samples in certain embodiments.

In a further specific embodiment, the panel of genes comprises, consists essentially of or consists of OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5. This embodiment applies in particular to tissue samples, which may be colon, rectal or appendix samples for example, as discussed herein.

In certain embodiments, the panel of genes comprises, consists essentially of or consists of OSMR, NDRG4, GATA5 and ADAM23, where blood based samples and in particular plasma or serum samples are utilised.

Thus, the invention provides a method of detecting a predisposition to, or the incidence of, early stage colorectal cancer and in particular stage 0 to II colorectal cancer in a blood sample, or derivative thereof such as a plasma or serum sample (preferably a plasma sample) comprising detecting an epigenetic change in at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, early stage colorectal cancer and in particular stage 0 to II colorectal cancer. This methodmay be applied to a panel consisting of these four genes.

It is noted that for each gene, it may be possible to detect an epigenetic change, in particular methylation of the gene, in a plurality of locations within the same gene. Thus, for example, a gene may incorporate more than one CpG island, or multiple sites within the same CpG island may be investigated as appropriate. As shown in the detailed description (experimental part) below, for example, OSMR can be assessed at two discrete locations, both providing useful diagnostically relevant results. The respective targets are designated herein as OSMR3 and OSMR4. In one embodiment, the panel of genes comprises, consists essentially of or consists of both OSMR3 and OSMR4. When OSMR is referred to herein, as for all other genes, reference is made to an investigation of an epigenetic change, in particular methylation which is relevant to colorectal cancer. Thus, the panels of genes in the present invention may incorporate assessment of multiple sites within the same gene as appropriate. Primers investigating multiple sites within the same genes are set forth in the tables above, see

particularly tables 2 to 18 (and especially tables 5 to 11 and 13 to 18).

As discussed in greater detail herein, the detection of an epigenetic change in each of the panel of genes may be carried out in a single reaction. Many suitable techniques allowing multiplexing are available and may be utilised in the present invention. Most depend upon use of suitable fluorescent molecules having distinguishable emission spectra. The skilled person can readily select from the many fluorophores available to determine which can be used in a multiplexing context.

In one embodiment, a universal quencher is utilised together with suitable fluorophore donors each having a distinguishable emission wavelength maximum. A particularly useful quencher is DABCYL. Together with a suitable quencher such as DABCYL the following fluorophores may each be utilised to allow multiplexing: Coumarin (emission maximum of 475nm), EDANS (491nm), fluorescein (515nm), Lucifer yellow (523nm), BODIPY (525nm), Eosine (543nm), tetramethylrhodamine (575nm) and texas red (615nm) (Tyagi et al., Nature Biotechnology, Vol. 16, Jan 1998; 49-53).

It is noted that the methylation status of additional genes may also be determined in order to supplement the methods of the invention. No gene has been found to be epigenetically silenced in every similar tumour. For this reason, it may be advantageous to target multiple DNA alterations to attain high rates of tumour detection. Thus, in one embodiment of the methods of the invention, the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily

gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is analysed in combination with the methylation status of at least one other gene involved in the establishment of cancer. The at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be combined with at least two other genes involved in the establishment of cancer. The at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) may be combined with and at least three, four, five or six other genes involved in the establishment of cancer. For colorectal cancer, the other genes involved in the establishment of cancer may be selected from the group consisting of SFRP1, SFRP2, GATA-4, GATA-5, CHFR, APC(2), MGMT, p16, Vimentin, p14, RASSF1a, RAB32, SEPTIN-9, RASSF2A, TMEFF2, NGFR or SMARCA3. However, any gene involved in the establishment of colorectal cancer may be utilized in combination with the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in the methods of present invention.

Genes that become methylated early in the process of carcinogenesis are not only ideal for screening purposes, but also interesting targets for early cancer detection and for monitoring the progression or outcome of cancers. In a further aspect, the invention provides for a method of cancer prognosis (prognosis to cancer) comprising detecting epigenetic silencing of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein epigenetic silencing of the gene is indicative for cancer development. Preferably, epigenetic silencing is detected by determination of the methylation status and/or measurement of expression level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). In one embodiment, the subject is suffering from advanced adenomas or at risk for developing AJCC stage I, II, III or IV cancer. In another embodiment, the outcome is the survival of the subject after a surgical resection, e.g. a noncurative or curative surgical resection.

Early detection of epigenetic silencing of one of more genes may provide justification for more definitive follow up of patients who have molecular, but not yet all the pathological or clinical, features associated with the malignancy. Identification of cancer at its earliest stage while it is still localized and readily treatable may

improve the clinical outcome in patients. Methods with a prognostic value should allow for the specific detection of tumours and not detect (benign) adenomas, and thus provide for a differential diagnosis between advanced adenoma versus benign adenoma. As shown in the detailed description (experimental part), gene promoter hypermethylation of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) was observed at a higher frequency in adenomas with concurrent colorectal cancer when compared to adenomas from patients that did not have colorectal cancer. This prognostic value is included within the definition of diagnosis.

In a related aspect, the invention provides a method for determining the histopathological stage of cancer and in particular gastrointestinal cancer, such as colorectal cancer in a sample comprising detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein detection of the epigenetic change is indicative of the histopathological stage of the cancer, such as colorectal cancer for example. All embodiments of the methods of the invention are hereby incorporated as appropriate and are not repeated for reasons of conciseness. The epigenetic change is generally one causing gene silencing. Preferably, epigenetic silencing is detected by determination of the

methylation status and/or measurement of expression levels of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) and the methylation status and/or expression level of the gene or genes is correlated to a histopathological stage of cancer. In this method, a sample is obtained from a subject suffering from, or suspected of suffering from any appropriate cancer in accordance with this invention, such as colorectal cancer for example. The methylation level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), the expression level of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), or a combination thereof is determined and correlated to a histopathological stage of the cancer. The "stage" of a cancer is a descriptor (usually numbers I to IV) of how much the cancer has spread. The stage often takes into account the size of a tumour, how deep it has penetrated, whether it has invaded adjacent organs, if and how many lymph nodes it has metastasized to, and whether it has spread to distant organs. Staging of cancer is important because the stage at diagnosis is the biggest predictor of survival, and treatments are often changed

based on the stage. As aforementioned, the description of suitable methods for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness.

In a specific embodiment, the invention provides a method for determining the histopathological stage of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto in a tissue or blood sample, or derivative thereof such as a plasma or serum sample. The most suitable genes and combinations of genes are described hereinabove for these specific test samples (at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC for blood samples for example) and are not repeated for reasons of conciseness.

In a further specific embodiment, the invention provides a method for predicting or monitoring progression of an adenoma (to a carcinoma), in particular in the context of gastrointestinal cancers such as colorectal cancer, comprising determining the methylation status of an NDRG2/NDRG4 subfamily gene and in particular the NDRG4 gene in a suitable test sample, wherein an elevated or increased level of methylation indicates that the adenoma is more likely to progress to a carcinoma (than if the level of methylation is lower). This embodiment applies particularly

to the region of the NDRG4 gene which is amplified using primer set 1 as set out in table **2** above. Thus, in one embodiment, these methods employ primer set 1 in order to determine the methylation status of the NDRG4 gene. As is discussed below, primer pair 1 allows distinguishing of adenomas that progress to cancer from those that will not progress. This is highly important for cancer screening. The test sample may be any suitable sample, as discussed extensively above. However, the sample is generally a suitable tissue sample, in particular an adenoma sample.

In a related embodiment, detecting increased levels of methylation towards the transcription start site of the NDRG4 gene may also be useful for monitoring the progression of cancer, and in particular gastrointestinal cancers such as colorectal cancer (CRC). As is shown herein, based upon the results obtained, it is predicted that spreading of methylation from more 5' regions of the promoter towards the transcription start site correlates with cancer progression (for example from adenoma to carcinoma). Thus, the invention provides a method for predicting or monitoring progression of a gastrointestinal cancer, such as CRC, comprising determining the methylation status of an NDRG2/NDRG4 subfamily gene in a suitable test sample, wherein an elevated or increased level of methylation towards the transcription start site indicates that the cancer is more progressed than if the level of methylation is lower. The transcription start site and promoter sequence are known from the published gene sequence information. Primer set 1 and 2 as defined herein may be utilised as appropriate in these methods. Primer set 1 is used to determine the methylation status of the NDRG4 gene

closer to the transcription start site than primer set 2. Thus, a comparison of such results may be useful in these methods.

As stated herein the methods of the invention for diagnostic, prognostic, or personalised medicinal care are preferentially used in connection with a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer. A number of techniques are currently available for detection of colorectal cancer. These include:

- Faecal occult blood tests (Guaiac and immunochemical)
- Colonoscopy and/or sigmoidoscopy
- X-ray after double-contrast barium enema or CT-colonography
- Faecal DNA test (PreGen-Plus®)

More accurate screening, surveillance of higher-risk patients and improved management of patient care may advantageously employ these existing methods and techniques to supplement the methods of the invention.

Faecal DNA testing is an emerging technology in screening for colorectal cancer. Pre-malignant adenomas and cancers shed DNA markers from their cells which are not degraded during the digestive process and remain stable in the stool. Capture, followed by amplification, for example using the Polymerase Chain Reaction, amplifies the DNA to detectable levels for assay. The faecal DNA integrity assay has been proposed as a useful tool for the detection of colorectal cancer. The presence of high-molecular-weight DNA fragments in stool is associated with colorectal cancer and may be

related to disease-associated differences in the regulation of proliferation and apoptosis. Detecting colorectal cancer by testing stool for DNA may alternatively be based on identifying oncogene mutations characteristic of colorectal neoplasia that are detectable in exfoliated epithelial cells in the stool. While neoplastic bleeding is intermittent, epithelial shedding is continuous, potentially making stool-based DNA testing (also known as fecal DNA [f-DNA]) testing more sensitive than other methods. Commercially available stool-based DNA tests for colorectal cancer include PreGen-Plus™ (EXACT Sciences Corporation, Marlborough, MA 01752 USA) which 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: adenomatous polyposis coli (APC), K-ras, and protein and molecular size 53,000 daltons (p53). This test is also designed to detect long DNA fragments, which have been specifically associated with cells called non-apoptotic colonocytes, which are common in CRC.

Accordingly, molecular screening of faecal samples focused on oncogene mutations and/or DNA integrity may complement the methods of present invention. In specific embodiments, the methods of the invention are used in combination with detecting DNA integrity, or at least one DNA oncogene mutation, or a combination of both detecting DNA integrity and at least one DNA oncogene mutation in the sample in order to detect a predisposition to, or the incidence of, colorectal cancer. The methods may be carried out on a

faecal sample. In one embodiment the method may also include the step of obtaining and/or processing the sample.

Testing can be performed diagnostically or in conjunction with a therapeutic regimen. Epigenetic loss of function of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) can be rescued by the use of DNA demethylating agents and/or DNA methyltransferase inhibitors. Testing can be used to determine what therapeutic or preventive regimen to employ on a patient and be used to monitor efficacy of a therapeutic regimen.

Accordingly, the invention also provides a method for predicting the likelihood of successful treatment of a cancer as defined herein and in particular gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein detection of the epigenetic change is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected. Alternatively, the method comprises measurement of expression levels of the at least

one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein a reduced level of expression indicates the likelihood of successful treatment of cancer is higher than if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is expressed at a higher level. For the avoidance of doubt it is stated that the description of suitable methods (sample types, cancer types, panels of genes etc.) for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness.

In an opposite scenario, 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 change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed

herein), wherein detection of the epigenetic change is indicative that the likelihood of resistance to treatment is lower than if the epigenetic modification is not detected.

Alternatively, the method comprises measurement of expression levels of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein a higher level of expression indicates the likelihood of resistance to treatment of cancer is higher than if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is expressed at a reduced level.

Thus, the patient population may be selected for treatment on the basis of their methylation status with respect to the relevant at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein - such as where the at least one gene is selected from GATA4, OSMR, NDRG4 and SFRP2 or selected from OSMR, NDRG4, GATA5 and ADAM23 where tissues or bodily fluid and in particular faecal or blood based samples and in particular plasma samples are utilised), which leads to down regulation of gene expression of the corresponding gene. This leads to a

much more focussed and personalised form of medicine and thus leads to improved success rates since patients will be treated with drugs which are most likely to be effective. The description of suitable methods for determining epigenetic silencing of the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness.

In certain aspects, epigenetic loss of function of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in adenoma can identify the need for treatment. Subjects having a disease such as colon neoplasia may be assayed for methylation of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein). Alternatively, the subject may be undergoing routine screening and may not necessarily be suspected of having a disease such as colon neoplasia. Detecting methylation of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in an adenoma can be used to improve sensitivity and/or specificity for detecting a colon neoplasia, since

such advanced adenoma may indicate that the probable course of the adenoma is development to a carcinoma. In such case, preventive treatment may be recommended and involve resection of the adenoma.

Accordingly, the invention provides a method for predicting suitable treatment of an adenoma obtained from a subject, comprising determining the methylation status of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in an adenoma, wherein if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is methylated, in particular hypermethylated, the need for treatment of the adenoma is identified. Preferably, the treatment comprises resection of the adenoma.

In an opposite scenario, the invention provides a method for predicting suitable treatment of an adenoma obtained from a subject, comprising determining the methylation status of at least one gene selected from at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in an adenoma, wherein if the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular

NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is unmethylated or methylated to a lesser degree, it is decided that there is no need of resection of the adenoma. The description of suitable methods for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness. The adenomas are typically of colonic origin in certain embodiments.

The invention further provides for a method of selecting a suitable treatment regimen for cancer or predisposition to cancer comprising determining epigenetic silencing of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the gene is epigenetically silenced, in particular hypermethylated or reduced expressed, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is selected for treatment.

In an opposite scenario, the invention provides for a method of selecting a suitable treatment regimen for cancer or predisposition to cancer comprising determing the methylation status and/or expression level of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the gene is unmethylated or higher expressed, treatment with a DNA demethylating agent and/or a DNA methyltransferase

inhibitor and/or a HDAC inhibitor is contra-indicated. Thus, alternative treatment should be explored.

In a related aspect, the invention also provides a method of selecting a suitable treatment regimen for cancer, in particular a gastrointestinal cancer such as colorectal cancer (as defined herein), comprising detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), wherein detection of the epigenetic change 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 change is not detected, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment. In the event that the epigenetic change is not detected (for example through gene expression detection or any other suitable method), alternative treatments should be explored. The description of suitable methods for determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness. In embodiments where blood and in particular plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4,

GATA5, ADAM23, JPH3, SFRP2 and APC. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23. Additionally or anternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. In addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3, such as from TFPI2, FOXE1, SYNE1, SOZ17, PHACTR3 and JAM3, in particular TFPI2. Suitable panels, as defined herein, are also envisaged, such as a panel comprising, consisting essentially of or consisting of OSMR, NDRG4, GATA4 and SFRP2 for example.

In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample.

In another aspect, the invention provides for a method of treating cancer and in particular colorectal cancer in a subject comprising administration of a DNA demethylating agent and/or a HDAC inhibitor and/or a DNA methyltransferase inhibitor wherein the subject has been selected for treatment on the basis of a method of the invention. Accordingly, the description of suitable methods for

determining epigenetic silencing of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) apply *mutatis mutandis* to these aspects of the invention and are not repeated here simply for reasons of conciseness. Thus, in embodiments where blood and in particular plasma or serum samples are utilised, the at least one gene may be selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. Suitable panels in this context comprise, consist essentially of or consist of OSMR, NDRG4, GATA5 and ADAM23.

Additionally or anternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, SYNE1 and SOX17, in particular TFPI2.

In embodiments where faecal samples are employed, the at least one gene may be selected from GATA4, OSMR, NDRG4, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT. In addition, or alternatively, the at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, and JAM3, such as from TFPI2, FOXE1, SYNE1, SOZ17, PHACTR3 and JAM3, in particular TFPI2. Suitable panels, as defined herein, are also envisaged, such as a panel comprising, consisting essentially of or consisting of OSMR, NDRG4, GATA4 and SFRP2 for example.

In embodiments in which tissue samples are utilised, the methods may comprise, consist essentially of or consist of

detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5. The tissue sample may comprise, consist essentially of or consist of a colon and/or rectal and/or appendix sample.

Thus, for the patient population where the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is methylated, which leads to decreased gene expression, this type of treatment is recommended. This method is referred to hereinafter as the "method of treatment" aspect of the invention.

In a related aspect, the invention also provides for the use of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor (in the manufacture of a medicament for use) in treating cancer, and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer in a subject, wherein the subject has been selected for treatment on the basis of the methods of the invention. Likewise, the invention provides a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor for use in treating cancer, and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer in a subject, wherein the subject has been selected for treatment on the basis of the methods of the invention.

For all of the relevant methods (pharmacogenetic methods, treatment regimen methods and methods of treatment) of the invention, the DNA demethylating agent may be any agent capable of up regulating transcription of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein - such as at least one gene selected from GATA4, OSMR, NDRG4, GATA5 and ADAM23). A preferred DNA demethylating agent comprises, consists essentially of or consists of a DNA methyltransferase inhibitor. The DNA methyltransferase inhibitor may be any suitable inhibitor of DNA methyltransferase which is suitable for treating cancer in the presence of methylation of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein - such as at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23). As is shown in the experimental section below, methylation of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein such as at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23) is linked to colorectal cancer and so preventing this methylation is predicted to help to treat a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer.

The DNA methyltransferase inhibitor may, in one embodiment, be one which reduces expression of DNMT genes, such as suitable antisense molecules, or siRNA 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 embodimens, 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).

In another embodiment, the DNA methyltransferase inhibitor comprises Decitabine. Full details of this drug can be found at www.supergen.com for example.

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).

Further agents which may alter DNA methylation and which may, therefore, be useful in the present compositions 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).

Useful DNMT inhibitors in the present invention comprise, consists essentially of or consists of 5-azacytidine and/or zebulaine.

As discussed above, one challenge faced by researchers investigating colorectal cancer is the diversity of DNA present in stool samples. The DNA of interest represents only a very small percentage of the total DNA isolated from stool. Therefore, along with the exploration of suitable DNA markers, techniques for improved DNA isolation and enrichment of the human DNA component from faecal samples are required for more sensitive cancer detection.