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

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

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

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

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

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

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

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

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

Claim Rejections - 35 USC § 103

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

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

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

<u>*Standard for Obviousness*</u>.

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

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

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

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

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

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

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

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

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

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

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

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

Admissions as Prior Art.

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

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

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

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

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

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

17. Claims 82-84, 87-98, and 100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.).

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

> Fecal occult blood tests fall primarily into two categories, tests based on the use of chromogenic chemical reagents such as gum guaiac and immunochemical tests. The chemically based guaiac methods determine the presence of occult blood by the detection

> of the perioxidase activity of the hemoglobin in the blood present in the faecal sample. They require catalysis of peroxide into oxygen and water, and the subsequent oxidation of a colorless dye (most often into a colored form). However, peroxidase activity is also found in meats and vegetables. In order to produce accurate results, these tests require restriction of the intake of certain foods, drugs, vitamins, and other substances prior to and during the sample collection period. The sensitivity of the most commonly used guaiac FOBT (Hemoccult) is approximately 50%. Despite a specificity of 98%, the positive predictive value for FOBT is low. Methods of detecting occult blood based on porphyrin (heme and protpoporphyrin IX) analysis or immunologic tests using anti-hemoglobin antibodies improve on these results**. Immunochemical tests (FIT or iFOBT) that use anti- hemoglobin antibodies specific for human blood in extracts from stool do not require dietary restrictions;** however, they are more complicated and more expensive than peroxidase-based tests. (Emphasis added)

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

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

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

> The methods of the invention may be carried out on purified or unpurified DNA-containing samples. However, in specific embodiments, DNA is isolated/extracted/purified from the sample. Any suitable DNA isolation technique may be utilized. Examples of purification techniques may be found in standard texts such as Molecular Cloning - A Laboratory Manual (Third Edition), Sambrook and Russell (see in particular Appendix 8 and Chapter 5 therein). (Emphasis added)

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

> Amplification of DNA (using PCR) from natural sources is often inhibited by co-purified contaminants and various methods adopted for DNA extraction from environmental samples are available and provide an alternative for isolating DNA from faecal samples, according to specific embodiments of the invention. For instance, the QIAamp DNA Stool Mini Kit from QIAGEN adsorbs DNA-damaging substances and PCR inhibitors

present in the sample by InhibitEX. Other examples for application to faecal samples include the Wizard Genomic DNA Purification Kit (Promega), the NucliSENS® easyMAG TM (Biomerieux) and nucleic acid purification kits manufactured by Macherey Nagel.

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

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

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

> **Determination of the methylation status may be achieved through any suitable means. Suitable examples include bisulphite genomic sequencing and/or by methylation specific PCR. Various techniques for assessing methylation status are known in the art and can be used in conjunction with the present invention**: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR(McMS-PCR), MLPA with or without bisulphite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulphite conversion-specific methylation-specific PCR (BS-MSP)(Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence diferences in PCR products of sodium bisulphite - treated DNA), methylation-sensitive single-nucleotide primer extension conformation(MS-SNuPE), methylation- sensitive single-strand conformation analysis (MS-SSCA), Melting curve combined bisulphite restriction analysis (McCOBRA)(Akey et al., 2002), PyroMethA, HeavyMethyl (Cottrell et al. 2004), MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles (QAMA), enzymatic regional

> methylation assay (ERMA), QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques for DNA methylation analysis is provided in Nucleic acids research, 1998, Vol. 26, No. 10, 2255-2264, Nature Reviews, 2003, Vol.3, 253-266; Oral Oncology, 2006, Vol. 42, 5- 13, which references are incorporated herein in their entirety. (Emphasis added)

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

> In the MSP approach, DNA may be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulphite treatment (**Herman et al., 1996; and WO 97/46705**). For example, bisulphite ions modify non-methylated cytosine bases, changing them to uracil bases. Uracil bases hybridize to adenine bases under hybridization conditions. (Emphasis added)

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

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

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

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

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

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

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

> A method for preparing a fecal sample for immunoassay testing comprising the steps of dispersing **a sample of from 1 to less than 10 wt. % of a stool sample** in an aqueous fecal test solution formulated with one or more preservatives, analyte stabilizing agents and endogenous interference reducing agents. The fecal solids are then permitted to settle to form a liquid phase substantially free from fecal solids, and the clarified liquid phase is removed to provide a test sample free from fecal solids. **The fecal test solutions contain suitable stool stabilizers such as buffers** and antimicrobial agents, analyte protecting agents such as proteolytic, reductive or oxidative enzyme inhibitors, endogenous assay interfering enzyme inhibitors such as a reducing agent, and non-specific binding inhibitors such as animal proteins. **The stool sample should be fresh or be fresh frozen** and thawed immediately before dispersion in the buffer solution. The sample is suitable for any solid-phase immunoassay determination of a fecal sample analyte. A method for determining analyte in the stool sample comprises conjugating anti-analyte antibody adhered to an insoluble support with analyte in the clarified sample, and determining the presence and extent of such conjugation. For determining human hemoglobin in a sample of human stool, the aqueous fecal test solution preferably contains a proteinase inhibitor, formaldehyde and an animal albumin. (Emphasis added)

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

of claim 82, step c). The fact that the sample can be frozen is deemed to meet a limitation of claim 83.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

45. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the teachings of applicant, Yee et al., Grow et al., and Shuber et al., whereby the gene to be evaluated in terms of methylation was FOXE1 as it had already been associated with cancer.

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

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

Conclusion

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

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

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

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

52. Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished

applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

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

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

U.S. PATENT DOCUMENTS

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

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

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

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

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

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

CLAIMS

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

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020						
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CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020						
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	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

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(54) Title: ABERRANTLY METHYLATED GENES IN PANCREATIC CANCER

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

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ABERRANTLY METHYLATED GENES IN PANCREATIC CANCER

RELATED APPLICATIONS

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

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

SUMMARY OF THE INVENTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

BRIEF DESCRIPTION OF THE FIGURES

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

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

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

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

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

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

DETAILED DESCRIPTION OF THE INVENTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

EXAMPLE 1

Materials and Methods

A. Collection and Preparation of Pancreatic Cell Lines

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

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

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

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

C. Oligonucleotide Array Hybridization

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

D. Reverse Transcription-PCR (RT-PCR)

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

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

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

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

F. Statistics

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

EXAMPLE 2

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

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

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

EXAMPLE 3

Identification of Genes Induced by TSA in Pancreatic Cancer Cell Lines

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

EXAMPLE 4

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

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

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

EXAMPLE 5

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

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

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

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

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

EXAMPLE 6

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

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

EXAMPLE 7

Methylation Analysis of Multiple Genes in Primary Pancreatic Cancers

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

EXAMPLE 8

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

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

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

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

EXAMPLE 9

Summary

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

TABLE 1

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

TABLE 2

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

TABLE 3

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

TABLE 4

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

TABLE 4 (continued)

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

What is claimed is:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

a) carrier means compartmentalized to receive a sample therein;

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

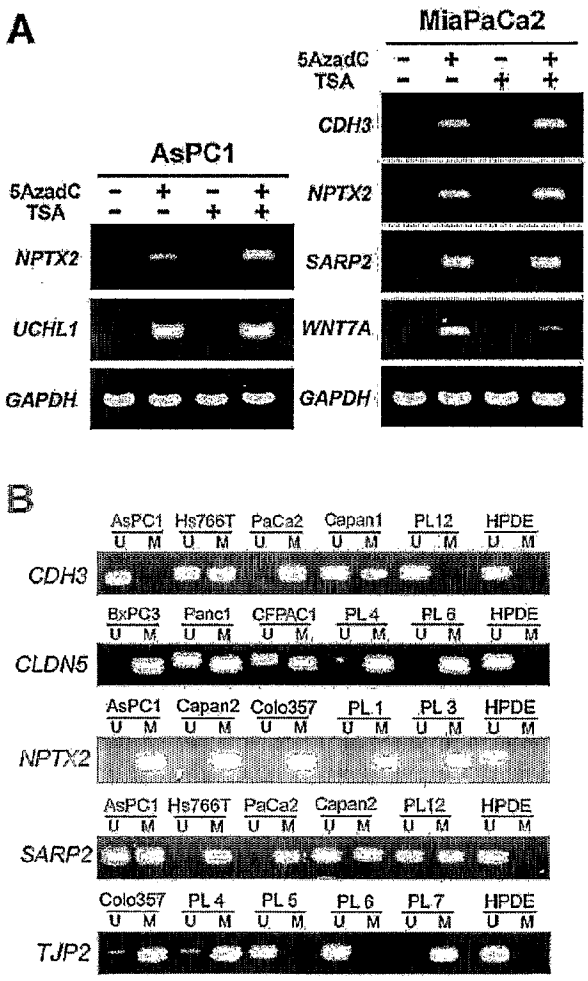

A
AsPC1
5AzadC
TSA
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UCHL1
GAPDH
MiaPaCa2
5AzadC
TSA
CDH3
NPTX2
SARP2
WNT7A
GAPDH
B
AsPC1 Hs766T PaCa2 Capan1 PL12 HPDE
CDH3
BxPC3 Panc1 CFPAC1 PL4 PL6 HPDE
CLDN5
AsPC1 Capan2 Colo357 PL1 PL3 HPDE
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SARP2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

Figure 1

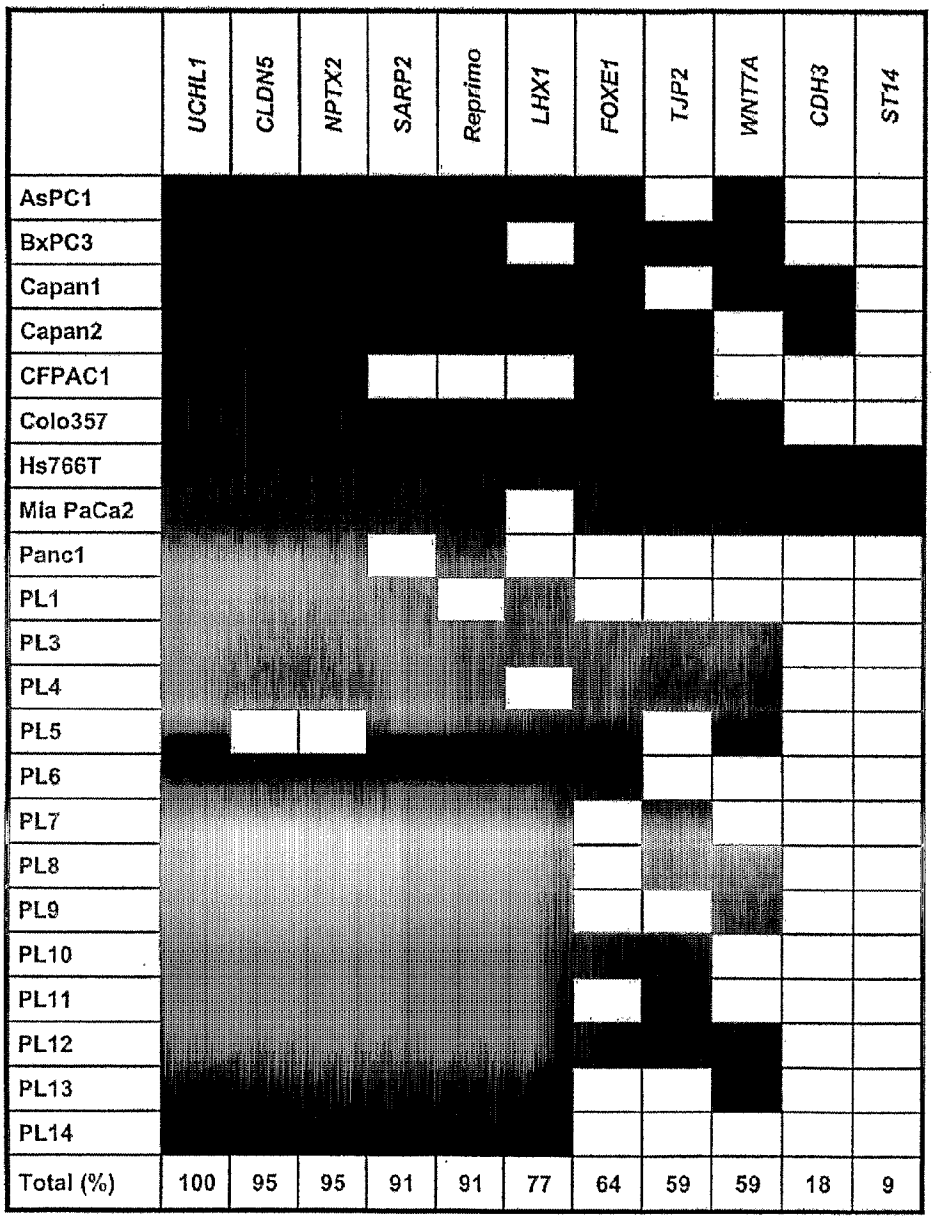

UCHL1
CLDN5
NPTX2
SARP2
Reprimo
LHX1
FOXE1
TJP2
WNT7A
CDH3
ST14
AsPC1
BxPC3
Capan1
Capan2
CFPAC1
Colo357
Hs766T
Mia PaCa2
Panc1
PL1
PL3
PL4
PL5
PL6
PL7
PL8
PL9
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59
59
18
9

Figure 2

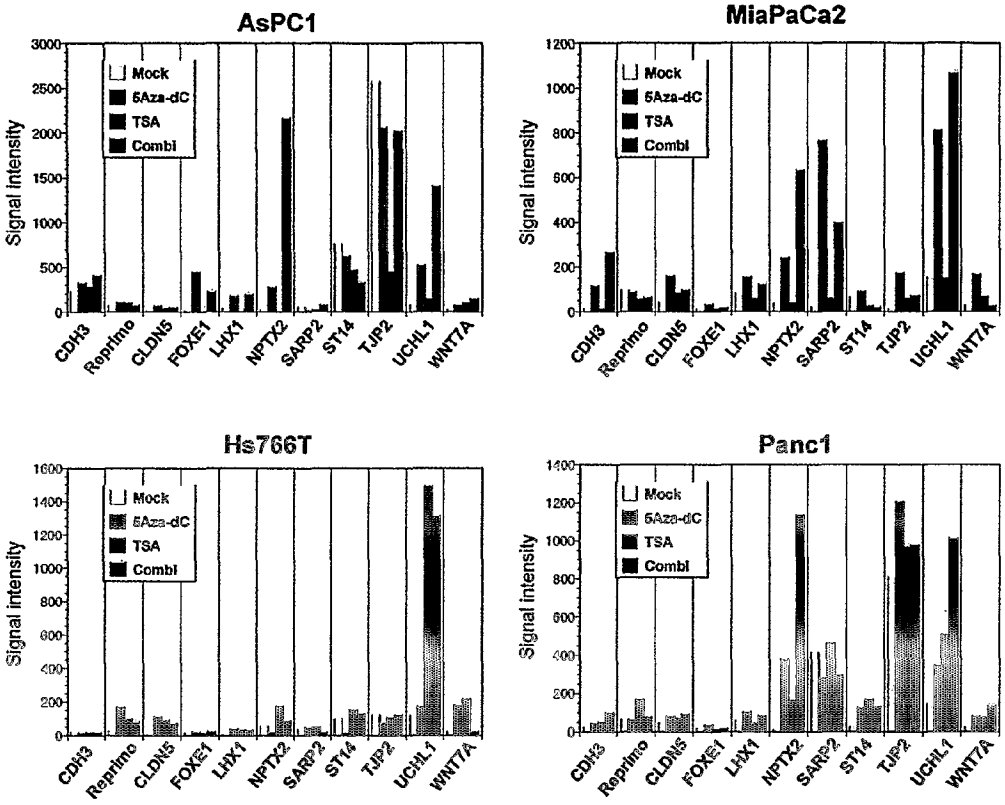

AsPC1
MiaPaCa2
Hs766T
Panc1
Mock
5Aza-dC
TSA
Combi
Signal intensity
CDH3
Reprimo
CLDN5
FOXE1
LHX1
NPTX2
SARP2
ST14
TJP2
UCHL1
WNT7A

Figure 3

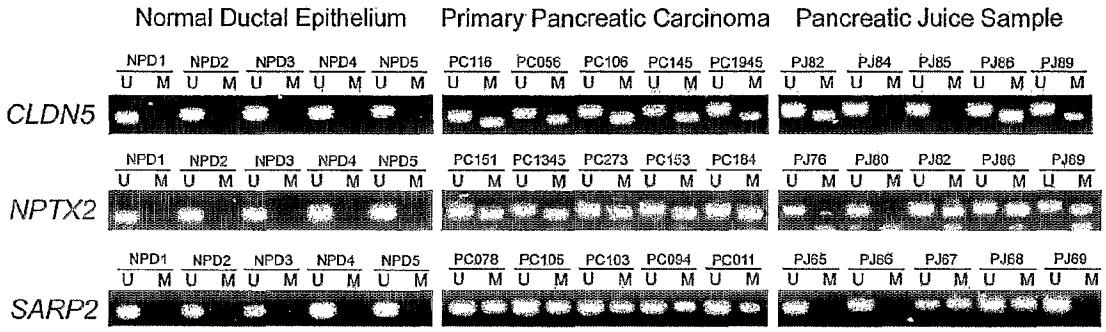

Normal Ductal Epithelium
Primary Pancreatic Carcinoma
Pancreatic Juice Sample
CLDN5
NPTX2
SARP2
NPD1 NPD2 NPD3 NPD4 NPD5
PC116 PC056 PC106 PC145 PC1945
PJ82 PJ84 PJ85 PJ86 PJ89
PC151 PC1345 PC273 PC153 PC184
PJ76 PJ80 PJ82 PJ86 PJ89
PC078 PC105 PC103 PC094 PC011
PJ65 PJ66 PJ67 PJ68 PJ69
U M

Figure 4

```
Connecting via Winsock to STN at stnc.cas.org on port 23


Welcome to STN International!  Enter x:X

LOGINID:SSSPTAU187BXS

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


* * * * * * * * * *   Welcome to STN International   * * * * * * * * * *

 NEWS  1  MAR  7  Search Limits Significantly Expanded Exclusively in STNext(R)
 NEWS  2  APR 18  New legal status codes for US patent applications and
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 NEWS  3  APR 30  Derwent Markush Resource (DWPIM) Database Enhancements
 NEWS  4  OCT  7  New CAS FORMULATIONS TM Database Launched on STNext
 NEWS  5  OCT  8  Third 2019 update to Emtree(TM) now available on STN(R)
 NEWS  6  NOV  8  Derwent Markush Resource (DWPIM) Enhanced to Improve Search
                  and Retrieval Quality
 NEWS  7  NOV 15  INPADOC Enhanced with More Current and Complete Japanese Legal
                  Status Information.
 NEWS  8  NOV 18  German Patent Full-Text Database DEFULL enhanced
 NEWS  9  NOV 25  CAS FORMULATIONS (TM) Enhanced on STNext
 NEWS 10  NOV 25  PatentPak(R) now available in USPATFULL/USPAT2 on STNext(R)
 NEWS 11  DEC 10  CAplus (SM) Now Provides Non-Conventional Patent Families
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 NEWS 12  DEC 23  Derwent World Patents Index: Manual Code Revision 2020

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=> File medline biosis
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                       0.18       0.18
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FILE 'MEDLINE' ENTERED AT 14:21:14 ON 14 JAN 2020

FILE 'BIOSIS' ENTERED AT 14:21:14 ON 14 JAN 2020
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=> s NDRG4 and (methylation or hypermethylation) and py<2009
L1            1 NDRG4 AND (METHYLATION OR HYPERMETHYLATION) AND PY<2009

=> t l1

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


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

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

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

=> d his

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

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

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

=> loy y
```

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

=> log y
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                       2.34      16.28

STN INTERNATIONAL LOGOFF AT 14:33:38 ON 14 JAN 2020
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Patent Owner's Ex. 2003, Page 447

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	2	(cancer carcinoma) same (PHACTR3 NDRG4 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFRP2 CDO1) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:40
L2	1	"200483399"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:42
L3	0	"200483399A2"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:43
L4	0	"200483399A2"	EPO	OR	OFF	2020/01/15 11:11
L5	0	"200483399"	EPO	OR	OFF	2020/01/15 11:11
S1	53	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:50
S2	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:51
S3	9	(hemoglobin haemoglobin) with concentration same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:18
S4	260	DNA same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:35
S5	2	"20080227208"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	OFF	2019/06/23 13:34

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			DERWENT; IBM_TDB			
S6	1	"20080227208" and "10 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:35
S7	11	(hemoglobin haemoglobin) with "5 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:38
S8	0	NDGR4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S9	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S10	6	NDRG4 same epigenetic and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:58
S11	24	"2006113770"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:02
S12	4	"2006113770" and NDRG4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:11
S13	6	NDRG4 same (epigenetic methylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S14	6	NDRG4 same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S15	0	"N-myc downstream regulated gene 4" same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	OFF	2020/01/14 14:24

Patent Owner's Ex. 2003, Page 449

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

EAST Search History (Interference)

< This search history is empty>

1/ 15/ 2020 11:51:40 AM

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15634607	CTNF	01/21/2020	EXCTD-35239.304
	892	01/21/2020	EXCTD-35239.304

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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

RESPONSE TO NON-FINAL OFFICE ACTION MAILED 01/21/2020

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

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

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

This paper is responsive to the Office Action mailed 01/21/2020, with response due by 04/21/2020. A one-month extension of time, to 05/21/2020, is requested. Applicant respectfully requests reconsideration in view of the remarks hereinbelow.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: **EXCTD-35239.304**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Remarks begin on Page 2.

REMARKS

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

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

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

The claims are not obvious

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

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

- That immunochemical assays for detecting human hemoglobin in fecal sample were known in the art (Office Action, ¶17);
- That detection of colorectal carcinoma based upon determining methylation of a number of different genes is disclosed in is disclosed in WO 2008/084219 Al (Van England et al.) (Office Action, ¶18);
- That methods for isolating DNA are known in the art. (Office Action, ¶19);
- That kits for amplifying DNA in stool samples are commercially available (Office Action, ¶20);
- That kits for isolating DNA from fecal samples are commercially available. (Office Action, ¶21);
- That numerous methods for assessing methylation are known in the art, including methods that comprise polymerase chain reaction (PCR), which is recognized as a form of amplifying DNA. Also admitted to therein is the use of chemical reagents so to modify the DNA prior to amplification was also well known in the art. (Office Action, ¶22);

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

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

Without acquiescing to the Examiner's characterization of the teachings of Yee, Grow, and Shuber, Applicant points out that none of Yee, Grow, or Shuber teach or suggest analysis of a single collected fecal sample for both hemoglobin and nucleic acid, much less testing hemoglobin in combination with the *NDRG4* nucleic acid marker of Applicant's elected species. Further, the combination of references and alleged admissions does not point to collecting and processing a fecal sample in the manner set forth in Applicant's claims 82-84 and 87-100, in which the sample is divided and the portions are separately treated to preserve blood proteins in a removed portion and to stabilize the remaining portion, *e.g.*, for extraction of DNA for analysis.

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

While *KSR* relaxed some of the formalism accompanying earlier decisions requiring a "teaching, suggestion, or motivation" to combine prior art references, it did not eliminate the requirement to anchor the nonobviousness calculus in an explanation of how a person of ordinary skill would select and apply the teachings of the cited art. See, e.g., *KSR*, 550 U.S. at 417. Rather, rejections on obviousness "cannot be sustained by mere conclusory statements; instead,

there must be some articulated reasoning with some rational underpinning" *KSR*, 550 U.S. at 418 (citing *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)), that one of skill in the art would have been motivated to combine the elements to yield the claimed combination. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991). See MPEP § 2143.

In making the rejection, the Examiner states that "In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to analyze fecal samples for both blood (hemoglobin) as well as for gene/sequence of interest." (Office Action, ¶35). The Examiner further asserts that "In view of the intense interest in detection of colorectal carcinoma in an individual, said ordinary artisan would have been amply motivated. And in view of the well-developed state of the art, and the fact that one would be using the disclosed methods for the same purpose, said ordinary artisan would have had a most reasonable expectation of success." (Office Action, ¶36). The Examiner provides no additional analysis or argument in support of making the combination.

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

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

Notably, however, neither the references nor the Examiner identify any deficits of blood/hemoglobin testing of fecal samples that are curable by the addition of Shuber's nucleic acid testing, nor vice versa. The references thus provide no suggestion that combining these methods of analysis, as urged by the Examiner, might offer any particular improvement or

benefit over the use of one testing method alone. Rather, the skilled person considering the cited art could only guess at whether expending the time and effort to conduct both blood/hemoglobin testing and nucleic acid testing on a fecal sample *might* produce some beneficial result, rather than merely producing redundant information.

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

While not acquiescing that other requirements for establishing prima facie obviousness have been met, for the reasons set forth above, Applicant submits that prima facie obviousness of claims 82-84 and 87-100 over Yee, Grow, and Shuber has not been established for at least the reason that the Examiner has relied on conclusory statements and has failed to establish as required that one of skill in the art would have been motivated to combine the elements to yield the claimed combination, and has further failed to establish that the skilled person would have a reasonable expectation success. Applicant therefore respectfully requests that the rejection of claims 82-84 and 87-100 be withdrawn.

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

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

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

Furthermore, Goggins fails to cure the deficiencies of the Examiner's arguments regarding Yee, Grow, and Shuber set forth above and, for the same reasons, these arguments fail to establish the obviousness of claim 99 in view of the combination of Yee, Grow, Shuber and Goggins. Applicant therefore respectfully requests that the rejection of claim 99 be withdrawn.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that all grounds for rejection have been addressed and Applicant's claims should be passed to allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicant encourages the Examiner to call the undersigned at (608) 662-1277.

Dated: May 21, 2020 /Mary Ann D. Brow /

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Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

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

Electronic Acknowledgement Receipt

EFS ID:	39513398
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow
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Attorney Docket Number:	EXCTD-35239.304
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	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Applicant Arguments/Remarks Made in an Amendment		2	6	
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30740 c0cab143aa6c76a3584398566da62c6fde4ec368	no	2
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
				Filing Date	06/27/2017
				First Named Inventor	LOUWAGIE
				Art Unit	1634
				Examiner Name	SISSON
Sheet	1	of	2	Attorney Docket Number	EXCTD-35239.304

U.S. PATENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	15/634,607
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				First Named Inventor	LOUWAGIE
				Art Unit	1634
				Examiner Name	SISSON
Sheet	2	of	2	*Attorney Docket Number*	EXCTD-35239.304

CERTIFICATION STATEMENT

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

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

OR

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☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

SIGNATURE

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

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

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Mini Review

Noninvasive molecular biomarkers for the detection of colorectal cancer

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

Introduction

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

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

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

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

Fecal markers

Fecal hemoglobin

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

Genes and epigenetic markers

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

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

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

Table 1. Noninvasive molecular biomarkers for the detection of CRC

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

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

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

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

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

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

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

Serum or blood markers

CEA

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

CA 19-9

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

Tissue inhibitor of metalloproteinase type 1

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

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

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

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

Nicotinamide N-methyltransferase and proteasome activator complex subunit 3

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

Collapsin response mediator protein-2

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

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

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

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

α-Defensins

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

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Macrophage migration inhibitory factor

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

Macrophage-colony stimulating factor

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

Prolactin

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

M2-pyruvate kinase

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

Methylated septin-9 DNA

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

Five gene markers in whole blood

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

Other markers

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

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

Conclusion and perspectives

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

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

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

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Fecal DNA–Based Detection of Colorectal Neoplasia

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

Rationale for Stool-Based Biomarkers for Detection of Colorectal Cancer

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

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

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

Cellular Trafficking in the Colonic Crypt

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

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

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

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

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

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

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

Multiplexed-DNA Mutation Panel for Human Stool

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

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

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

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

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

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

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

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

DNA Methylation-Based Stool Biomarkers

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

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

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

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

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

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

Conclusions

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

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

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•• Of major importance

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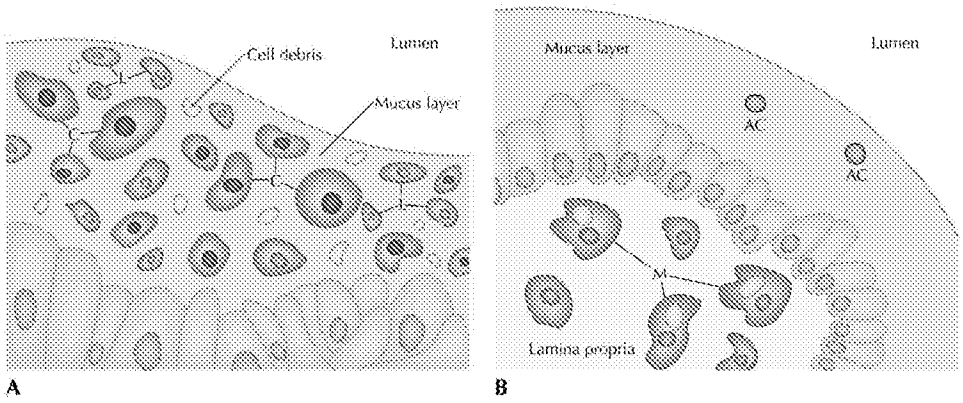


Figure 1.

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

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Table 1

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

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

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

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

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

Electronic Acknowledgement Receipt

EFS ID:	39936127
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	72960
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	07-JUL-2020
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	2020-07-07_35239-304_SupplIDS-SB08.pdf	151785 967b30010df51b226ae1ba339867e168057257b2	no	3
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	KIM.pdf	219952 1b535ec52a256030c047834395c29b165ca1cc98	no	8
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3	Non Patent Literature	TURGEON.pdf	835611 faa162e10aaafed00b2701cc76556db002eb237f	no	11
Warnings:					
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4	Fee Worksheet (SB06)	fee-info.pdf	30695 648372c2bab2b66ae1f12d4db3ab870bb210e048	no	2
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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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.

New International Application Filed with the USPTO as a Receiving Office

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

PAIR Correspondence Address/Fee Address Change

The following fields have been changed to Customer Number 173254 on 08/03/2020 via Private PAIR in view of the certification copied below that authorized the change.

- Correspondence Address

The address for Customer Number 173254 is:
173254
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

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

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

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

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

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

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

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

173254 7590 08/10/2020

CASIMIR JONES, S.C.
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EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
08/10/2020	ELECTRONIC

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

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

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

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

Office Action Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Bradley L Sisson	Art Unit 1634	AIA (FITF) Status No

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

Period for Reply

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

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

Status

1)☑ Responsive to communication(s) filed on 05/22/2020 & 07/07/2020.

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

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

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

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

Disposition of Claims*

5) ☑ Claim(s) 82-84 and 87-100 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 82-84 and 87-100 is/are rejected.

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

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

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

Application Papers

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

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

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

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

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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

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

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

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

Attachment(s)

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

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 07/07/2020.

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

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

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

Claim Interpretation

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

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

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

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

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

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

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

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

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

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

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

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

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

Claim Rejections - 35 USC § 103

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

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

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

Standard for Obviousness.

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

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

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

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

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

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

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

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

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

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

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

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

Admissions as Prior Art.

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

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

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

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

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

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

17. Claims 82-84, 87-98, and 100 remain rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.).

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

> Fecal occult blood tests fall primarily into two categories, tests based on the use of chromogenic chemical reagents such as gum guaiac and immunochemical tests. The chemically based guaiac methods determine the presence of occult blood by the detection

> of the perioxidase activity of the hemoglobin in the blood present in the faecal sample. They require catalysis of peroxide into oxygen and water, and the subsequent oxidation of a colorless dye (most often into a colored form). However, peroxidase activity is also found in meats and vegetables. In order to produce accurate results, these tests require restriction of the intake of certain foods, drugs, vitamins, and other substances prior to and during the sample collection period. The sensitivity of the most commonly used guaiac FOBT (Hemoccult) is approximately 50%. Despite a specificity of 98%, the positive predictive value for FOBT is low. Methods of detecting occult blood based on porphyrin (heme and protpoporphyrin IX) analysis or immunologic tests using anti-hemoglobin antibodies improve on these results**. Immunochemical tests (FIT or iFOBT) that use anti- hemoglobin antibodies specific for human blood in extracts from stool do not require dietary restrictions;** however, they are more complicated and more expensive than peroxidase-based tests. (Emphasis added)

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

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

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

> The methods of the invention may be carried out on purified or unpurified DNA-containing samples. However, in specific embodiments, DNA is isolated/extracted/purified from the sample. Any suitable DNA isolation technique may be utilized. Examples of purification techniques may be found in standard texts such as Molecular Cloning - A Laboratory Manual (Third Edition), Sambrook and Russell (see in particular Appendix 8 and Chapter 5 therein). (Emphasis added)

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

> Amplification of DNA (using PCR) from natural sources is often inhibited by co-purified contaminants and various methods adopted for DNA extraction from environmental samples are available and provide an alternative for isolating DNA from faecal samples, according to specific embodiments of the invention. For instance, the QIAamp DNA Stool Mini Kit from QIAGEN adsorbs DNA-damaging substances and PCR inhibitors

present in the sample by InhibitEX. Other examples for application to faecal samples include the Wizard Genomic DNA Purification Kit (Promega), the NucliSENS® easyMAG TM (Biomerieux) and nucleic acid purification kits manufactured by Macherey Nagel.

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

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

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

> **Determination of the methylation status may be achieved through any suitable means. Suitable examples include bisulphite genomic sequencing and/or by methylation specific PCR. Various techniques for assessing methylation status are known in the art and can be used in conjunction with the present invention**: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR(McMS-PCR), MLPA with or without bisulphite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulphite conversion-specific methylation-specific PCR (BS-MSP)(Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence diferences in PCR products of sodium bisulphite - treated DNA), methylation-sensitive single-nucleotide primer extension conformation(MS-SNuPE), methylation- sensitive single-strand conformation analysis (MS-SSCA), Melting curve combined bisulphite restriction analysis (McCOBRA)(Akey et al., 2002), PyroMethA, HeavyMethyl (Cottrell et al. 2004), MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles (QAMA), enzymatic regional

> methylation assay (ERMA), QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques for DNA methylation analysis is provided in Nucleic acids research, 1998, Vol. 26, No. 10, 2255-2264, Nature Reviews, 2003, Vol.3, 253-266; Oral Oncology, 2006, Vol. 42, 5- 13, which references are incorporated herein in their entirety. (Emphasis added)

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

> In the MSP approach, DNA may be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulphite treatment (**Herman et al., 1996; and WO 97/46705**). For example, bisulphite ions modify non-methylated cytosine bases, changing them to uracil bases. Uracil bases hybridize to adenine bases under hybridization conditions. (Emphasis added)

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

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

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

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

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

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

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

> A method for preparing a fecal sample for immunoassay testing comprising the steps of dispersing **a sample of from 1 to less than 10 wt. % of a stool sample** in an aqueous fecal test solution formulated with one or more preservatives, analyte stabilizing agents and endogenous interference reducing agents. The fecal solids are then permitted to settle to form a liquid phase substantially free from fecal solids, and the clarified liquid phase is removed to provide a test sample free from fecal solids. **The fecal test solutions contain suitable stool stabilizers such as buffers** and antimicrobial agents, analyte protecting agents such as proteolytic, reductive or oxidative enzyme inhibitors, endogenous assay interfering enzyme inhibitors such as a reducing agent, and non-specific binding inhibitors such as animal proteins. **The stool sample should be fresh or be fresh frozen** and thawed immediately before dispersion in the buffer solution. The sample is suitable for any solid-phase immunoassay determination of a fecal sample analyte. A method for determining analyte in the stool sample comprises conjugating anti-analyte antibody adhered to an insoluble support with analyte in the clarified sample, and determining the presence and extent of such conjugation. For determining human hemoglobin in a sample of human stool, the aqueous fecal test solution preferably contains a proteinase inhibitor, formaldehyde and an animal albumin. (Emphasis added)

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

of claim 82, step c). The fact that the sample can be frozen is deemed to meet a limitation of claim 83.

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

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

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

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

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

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

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

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

Response to argument

39. Applicant's representative, at pages 2-5 of the response of 22 May 2020, hereinafter the response, traverses the rejection of claims 82-84, 87-98, and 100 under pre-AIA 35 U.S.C. §103(a) as being unpatentable over applicant's admissions in view of US 2008/0227208 A1 (Yee et al.), US 5,198,365 (Grow et al.), and US 2008/0124714 A1 (Shuber et al.). At page 4 of the response said representative asserts:

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

40. The above argument has been considered and has not been found persuasive as applicant has not addressed all of the references relied upon. In particular, applicant has not addressed the teachings of the primary reference- applicant's own admissions as to what was known and how commercially-available kits can be used for the preparation of samples. It is further noted that

Yee et al., and grow et al., were not being relied upon for teaching a method of isolation. Rather, Yee et al., was being relied upon for teaching the setting of 10 ng/ml of hemoglobin in blood as a threshold value, and how such a teaching meets a limitation of claims 87-94. Grow et al., was being relied upon for teaching the freezing of a sample. Shuber et al., was relied upon for teaching methods for stabilizing biological samples for nucleic acid analysis wherein one uses a stabilizing buffer and can also freeze the sample.

41. While argument has been asserted that the amount of work required would be increased, no evidence has been provided to support this conclusion. To the contrary, and as noted above in paragraph 20, applicant asserts that "Any suitable DNA isolation technique may be utilized." (Emphasis added)

42. In view of the above analysis and in the absence of convincing evidence to the contrary, the rejection is maintained.

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

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

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

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

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

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

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

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

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

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

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

50. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the teachings of applicant, Yee et al., Grow et al., and Shuber et al., whereby the gene to be evaluated in terms of methylation was FOXE1 as it had already been associated with cancer.

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

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

Response to argument

53. At page 5, bridging to page 6 of the response said representative traverses the rejection of claim 99 under §103(a). As asserted to therein:

> Nothing in Goggins suggests any utility in testing fecal samples using both blood/hemoglobin testing *and* nucleic acid testing for the FOXE1 gene for pancreatic cancer...

54. The above argument has been considered and has not been found persuasive for as noted above, Goggins et al., teach that the cell sample can be obtained from a variety of sources, including blood, pancreas, and colon. In view of the explicit guidance to obtain cells and materials from such a variety of sources, and to evaluate known informative genes for epigenetic modifications that are informative, said ordinary artisan would have been both amply motivated and would have had a most reasonable expectation of success.

55. At page 6 of the response said representative asserts:

> Furthermore, Goggins fails to cure the deficiencies of the Examiner's arguments regarding Yee, Grow, and Shuber set forth above and, for the same reasons, these arguments fail to establish the obviousness of claim 99 in view of the combination of Yee, Grow, Shuber and Goggins.

The above argument has been considered and has not been found persuasive towards the withdrawal of the rejection for as noted above, the rejection has been maintained.

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

Conclusion

57. The prior art made of record and not relied upon is considered pertinent to applicant's disclosure.

a. US 2006/0084054 A1 (Alsobrook et al.), at paragraph [1531], teaches:

> Recently, **NDRG4**, another memember [*sic*] of Ndr family, was shown to be expressed in neurons of the brain and spinal cord. Its expression was markedly decreased in the brain of Alzheimer's disease patient (Zhou R H, Kokame K, Tsukamoto Y, Yutani C, Kato H, Miyata T. (2001) Characterization of the human NDRG gene family: a newly identified member, NDRG4, is specifically expressed in brain and heart. Genomics 73(1):86-97). Therapeutic modulation of this gene, expressed protein and/or use of antibodies or small molecule drugs targeting the gene or gene product are useful in the treatment of central nervous system disorders such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, schizophrenia and depression.

58. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

59. A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

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

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

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

63. 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 https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

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

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

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20080227208-A1	09-2008	Yee; Hsiao-Ching	G01N33/72	436/66
*	B	US-5198365-A	03-1993	Grow; Michael A.	G01N33/528	436/17
*	C	US-20080124714-A1	05-2008	Shuber; Anthony P.	C12Q1/6806	435/6.16
*	D	US-20060084054-A1	04-2006	Alsobrook; John	C07K14/47	435/6.14
	E					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
*	N	WO-2004083399-A2	09-2004	WO	Goggins 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 MM-YYYY format are publication dates. Classifications may be US or foreign.

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

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

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

Search Notes		
Search Notes	Date	Examiner
EAST (see transcript for databases and search terms)	06/23/2019	B. L. S.
Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.
EAST (see transcript for databases and search terms)	01/14, 15/2020	B. L. S.
STN (see transcript for databases and search terms)	01/14/2020	B. L. S.
EAST (updated)	08/04/2020	B. L. S.

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

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

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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CLAIMS

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

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020					
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CLAIM		DATE								
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	93	÷	✓	✓	✓					
	94	÷	✓	✓	✓					

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

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020					
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	97	÷	✓	✓	✓					
	98	÷	✓	✓	✓					
	99	÷	✓	✓	✓					
	100	÷	✓	✓	✓					

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

U.S. PATENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

U.S. PUBLISHED PATENT APPLICATIONS

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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]

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

Examiner Signature	/Bradley L. Sisson/	Date Considered	08/04/2020

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

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)	2020-07-07
Name/Print	Mary Ann D. Brow	Registration Number	42363

PTO Notes regarding this form:

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

[3] Applicant's unique citation designation number (optional).
[4] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04.
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[6] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document.
[7] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible.
[8] Applicant is to place a check mark here if English language Translation is attached.
This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**.

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CONFIRMATION NO. 1874

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/634,607	06/27/2017 RULE	435	1634	EXCTD-35239.304

APPLICANTS

EXACT SCIENCES DEVELOPMENT COMPANY, LLC, Madison, WI;

INVENTORS

Joost Louwagie, Dornach, SWITZERLAND;

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

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

**** FOREIGN APPLICATIONS ***************************

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

07/12/2017

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

ADDRESS

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TITLE

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/08/04 19:10
S1	53	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:50
S2	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:51
S3	9	(hemoglobin haemoglobin) with concentration same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:18
S4	260	DNA same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:35
S5	2	"20080227208"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:34
S6	1	"20080227208" and "10 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:35
S7	11	(hemoglobin haemoglobin) with "5 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:38
S8	0	NDGR4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR;	OR	OFF	2020/01/14 13:50

			FPRS; EPO; JPO; DERWENT; IBM_TDB			
S9	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S10	6	NDRG4 same epigenetic and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:58
S11	24	"2006113770"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:02
S12	4	"2006113770" and NDRG4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:11
S13	6	NDRG4 same (epigenetic methylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S14	6	NDRG4 same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S15	0	"N-myc downstream regulated gene 4" same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S16	0	"N-myc downstream regulated gene 4" and (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S17	0	"N-myc downstream regulated gene 4" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:25

S18	1	"20060019256" and uptg	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:55
S19	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 15:35
S20	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:07
S21	3	fecal same (antibody antibodies immunoassay) same (DNA RNA polynucleotide "nucleic acid" gene) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:26
S22	2	(cancer carcinoma) same (PHACTR3 NDRG4 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFRP2 CDO1) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:40
S23	1	"200483399"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:42
S24	0	"200483399A2"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:43
S25	0	"200483399A2"	EPO	OR	OFF	2020/01/15 11:11
S26	0	"200483399"	EPO	OR	OFF	2020/01/15 11:11
S27	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/08/04 18:14

EAST Search History (Interference)

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8/4/2020 7:19:42 PM
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From: PAIR_eOfficeAction@uspto.gov
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MIDDLETON, WI 53562
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
15634607	CTFR	08/10/2020	EXCTD-35239.304
	892	08/10/2020	EXCTD-35239.304
	1449	08/10/2020	EXCTD-35239.304

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Docket Number (Optional)
EXCTD-35239.304

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Signature /Mary Ann D. Brow/

Typed or printed name Mary Ann D. Brow, Reg. No. 42363

In re Application of
LOUWAGIE

Application Number	Filed
15/634,607	27-Jun-2017

For
FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Art Unit	Examiner
1634	SISSON

Applicant hereby **appeals** to the Patent Trial and Appeal Board from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 840

☐ Applicant asserts small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by 50%, and the resulting fee is: $ _____

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I am the

☐ applicant

☑ attorney or agent of record
Registration number 42363

☐ attorney or agent acting under 37 CFR 1.34
Registration number _____

Signature /Mary Ann D. Brow/

Typed or printed name Mary Ann D. Brow

Telephone Number 608-662-1277

Date 11/10/2020

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Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
NOTICE OF APPEAL	1401	1	840	840
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**840**

Electronic Acknowledgement Receipt

EFS ID:	41082896
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	173254
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	10-NOV-2020
Filing Date:	27-JUN-2017
Time Stamp:	16:56:22
Application Type:	Utility under 35 USC 111(a)

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1	Notice of Appeal Filed	2020-11-10_35239-304_Not_Appeal.pdf	255260 a9d78aed3ffa2299c75d97756f48b28d24718405	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30708 01022fe414b5a5a996848377fa834821a6e107c1	no	2
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New International Application Filed with the USPTO as a Receiving Office

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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPELLANT'S BRIEF

APPEAL NO.:

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

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Dated: March 9, 2021 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This Brief supports the appeal to the Patent Trial and Appeal Board from the Final Office Action dated 08/10/2020 (hereinafter "Office Action") in the application identified above, and is in furtherance of the Notice of Appeal filed 11/10/2020, with Appellant's Brief due by 01/10/2021. A two-month extension of time, to 03/10/2021, is requested.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: **EXCTD-35239.304**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

This Brief contains these items under the following headings and in the order set forth below 37 C.F.R. § 41.37:

I. REAL PARTY IN INTEREST 3
II. RELATED APPEALS AND INTERFERENCES 3
III. SUMMARY OF CLAIMED SUBJECT MATTER 3
IV. ARGUMENT 7
V. CLAIMS APPENDIX 22

I. REAL PARTY IN INTEREST

The real party in interest is Exact Sciences Corp., Madison, WI., with subsidiary and Assignee of the application, Exact Sciences Development Company, LLC., Madison, WI.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to Appellant or Appellant's legal representative.

III. SUMMARY OF CLAIMED SUBJECT MATTER

Claims 1-81 and 85-86 were previously cancelled and claims 82-84 and 87-100 are pending, with claims 82 and 87 being independent. In response to the Office's requirement to elect one specific gene from claim 99, Appellant elected NDRG4.

The pending claims are directed to a method of processing a human fecal sample, *e.g.*, from a patient, in preparation for testing a single fecal sample for the presence of blood in the sample and also for the presence of epigenetic modification of DNA contained in the sample. (See, *e.g.*, the Specification of the instant application at page 28, first full paragraph.)

Claim 82 is directed toward a method of processing a fecal sample, the method comprising:

a) collecting a fecal sample from a human subject; (*see, e.g.,* Specification, page 32 at line 5)

b) removing a portion of said fecal sample to produce a removed portion and a remaining portion of said fecal sample; *(see, e.g., id.* at page 5, last full paragraph, and page 28, second full paragraph)

c) combining the removed portion with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample; *(see, e.g., id.* at page 5, last full paragraph, and page 28, second full paragraph)

d) stabilizing the remaining portion of the fecal sample. *(see, e.g., id.* at page 5, last full paragraph, and page 28, second full paragraph)

Claim 83 is directed to the method of claim 82, wherein stabilizing of the remaining portion of the fecal sample comprises one or both of:

i) adding a stabilizing buffer to said remaining sample; *(see, e.g., id.* at page 28, second full paragraph

ii) freezing said remaining sample. *(see, e.g., id.* at page 18, last paragraph)

Claim 84 is directed to method of claim 82, further comprising delivering the removed portion and the remaining portion of the fecal sample to a medical diagnostics laboratory. *(see, e.g., id.* at page 28, last paragraph)

85-86 were previously cancelled.

Claim 87 is directed to a method of processing a fecal sample, the method comprising:

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

B) testing the removed portion of said fecal sample for the presence of blood. *(see, e.g., id.* at page 28, last paragraph)

Claim 88 is directed to the method of claim 87, wherein said testing comprises testing the removed portion of said fecal sample for a concentration of hemoglobin present in said removed portion. *(see, e.g., id.* at page 12, first full paragraph)

Claim 89 is directed to the method of claim 87, wherein testing for the concentration of hemoglobin in said removed portion comprises immunochemical detection of hemoglobin. *(see, e.g., id.* at page 12, first full paragraph)

Claim 90 is directed to the method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml. *(see, e.g., id.* at paragraph bridging page 13-

14)

Claim 91 is directed to the method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml. *(see, e.g., id.* at paragraph bridging page 13-14)

Claim 92 is directed to the method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml. *(see, e.g., id.* at paragraph bridging page 13-14)

Claim 93 is directed to the method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 50 ng/ml. *(see, e.g., id.* at paragraph bridging page 13-14)

Claim 94 is directed to the method of claim 88, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml. *(see, e.g., id.* at paragraph bridging page 13-14)

Claim 95 is directed to the method claim 87, further comprising:

C) extracting DNA from the stabilized remaining portion of said fecal sample *(see, e.g., id.* at page 17, last full paragraph) and

D) analyzing said DNA for the presence of DNA from a human, wherein the DNA from a human has an epigenetic modification. . *(see, e.g., id.* at page 14, first full paragraph)

Claim 96 is directed to the method of claim 95, wherein said epigenetic modification comprises aberrant methylation. *(see, e.g., id.* at page 20, second paragraph)

Claim 97 is directed to the method of claim 95, wherein said aberrant methylation comprises hypermethylation. *(see, e.g., id.* at page 20, second paragraph)

Claim 98 is directed to the method of claim 95, wherein said DNA from a human comprises a gene and/or a promoter region of a gene. *(see, e.g., id.* at page 20, second paragraph)

Claim 99 is directed to the method of claim 98, wherein said gene selected from the group consisting of PHACTR3, NDRG4, FOXE1 , GATA4, GPNMB, TFPI2, SOX17, SYNE1 , LAMA, MMP2, OSMR, SFRP2, and CDO1. *(see, e.g., id.* at page 37, third paragraph)

Claim 100 is directed to the method of claim 96, wherein analyzing said DNA comprises modifying said DNA with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil. *(see, e.g., id.* at page 23, second paragraph)

IV. ARGUMENT

The Argument is set forth in the following sections:

A. Grounds of rejection to be reviewed;

B. Summary of the technology and arguments;

C. Summary of teachings cited and characterized by the Office;

D. Applicable law; and

E. Detailed argument.

A. Grounds of rejection to be reviewed

i. Whether the Office erred in rejecting claims 82-84 and 87-100 under pre-AIA 35 U.S.C. §103(a) as being unpatentable over Appellant's alleged admissions in view of US 2008/0227208 A1 ("Yee"); US 5,198,365 ("Grow"); and US 2008/0124714 A1 ("Shuber").

ii. Whether the Office erred in rejecting claim 99 as being unpatentable over Appellant's alleged admissions in view of Yee, Grow, and Shuber, as applied to claim 82, further in view of WO 2004/083399 A2 ("Goggins").

B. Summary of the technology and arguments

The instant claims are directed to a method of collecting a fecal sample from a subject and dividing the collected sample into portions that are separately stabilized, *e.g.*, for detection of different types of analytes in each portion. In particular embodiments, one portion is treated to stabilize blood proteins and another portion is separately treated to stabilize nucleic acid, *e.g.*, DNA. A fecal sample collected in the claimed manner is suitable for detecting blood proteins in one portion and DNA in the other portion of the same fecal sample.

The Office has erred in finding the claims obvious for at least the following reasons:

1) The Office has mischaracterized the teachings of Grow, and all rejections rely on conclusions drawn from this mischaracterization;

2) The combination of references fails to teach all the features of the invention as claimed; and

3) The Office relied on conclusory statements to support the rejection and has failed to provide articulated reasoning with proper rational underpinning to support the legal conclusion of obviousness, and has relied on improper hindsight reconstruction to arrive at Appellant's claims.

C. Summary of teachings cited and characterized by the Office

Appellant's Specification ("Appellant's alleged admissions")

In making the rejections, the Office starts with Appellant's alleged admissions as the primary basis for the rejections. The Office points to Appellant's Specification as allegedly teaching the following list of features:

- That immunochemical assays for detecting human hemoglobin in fecal sample were known in the art (Office Action, ¶18);
- That detection of colorectal carcinoma based upon determining methylation of a number of different genes is disclosed in is disclosed in WO 2008/084219 Al (Van England et al.) (Office Action, ¶19);
- That methods for isolating DNA are known in the art. (Office Action, ¶20);
- That kits for amplifying DNA in stool samples are commercially available (Office Action, ¶21);
- That kits for isolating DNA from fecal samples are commercially available. (Office Action, ¶22);
- That numerous methods for assessing methylation are known in the art, including methods that comprise polymerase chain reaction (PCR), which is recognized as a form of amplifying DNA. Also admitted to therein is the use of chemical reagents so to modify the DNA prior to amplification was also well known in the art. (Office Action, ¶23);
- That methods for performing MSP [methylation-specific PCR], wherein one employs primer pairs designed to distinguish methylated DNA from unmethylated DNA, was known in the art. (Office Action, ¶24); and

– That:

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

(Office Action, ¶26).

The Office admits that Appellant's disclosure does not teach that using a concentration of 10 ng/ml of hemoglobin in stool as a threshold value or freezing a portion of a fecal sample were know in the art (Office Action, ¶¶27 and 29.)

Cited secondary references

In making the rejections, the Office further points to four secondary references: Yee, Grow, Shuber, and Goggins.

1. The Office points to Yee as teaching setting a 10 ng/ml of hemoglobin in blood as a threshold value (Office Action, ¶¶28 and 40.)

2. The Office points to Grow as teaching using 1 to less than 10% of a sample preparation of a fecal sample for immunoassay, and for freezing a sample (Office Action, ¶¶ 30-32, and 40.)

3. The Office points to Shuber as teaching treating a stool sample with a nucleic acid stabilizing solution for nucleic acid analysis and for freezing a sample. (Office Action, ¶¶33-35 and 40.)

4. The Office points to Goggins as teaching testing FOXE1 in association with pancreatic carcinoma, and that neoplasm can occur in the colon. (Office Action, ¶¶ 46-49.)

D. Applicable Law

A proper obviousness determination requires that an Office make "a searching comparison of the claimed invention – *including all its limitations* – with the teaching of the prior art." *See In re Wada and Murphy*, Appeal 2007-3733, *citing In re Ochiai*, 71 F.3d 1565, 1572 (Fed. Cir. 1995) (emphasis in original). Further, the necessary presence of all claim features is axiomatic, since the Supreme Court has long held that obviousness is a question of law based on underlying factual inquiries, including … ascertaining the differences between *the claimed invention* and the prior art. *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966) (emphasis added). Indeed, Section 904 of the MPEP instructs Examiners to conduct an art search that covers "the invention *as described and claimed*." In short, it remains well-settled law that obviousness requires at least a suggestion of all of the features in a claim. *See In re Wada and Murphy*, *citing CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) and *In re Royka*, 490 F.2d 981, 985 (CCPA 1974)).

MPEP § 2143 points to the Supreme Court in *KSR Int'l v. Teleflex Inc.,* 127 S. Ct. 1727 (2007) as stating that "[r]ejections on obviousness grounds **cannot be sustained by mere conclusory statements**; instead, **there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness**." *KSR Int'l v. Teleflex Inc.*, 127 S. Ct. 1727, 1741 (2007) (*quoting In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)(emphasis added).

It has further been confirmed by the Board in *In re Nuvasive, Inc.*, 842 F.3d 1376 (2016), that "**conclusory statements" alone are insufficient** and, instead, the finding **must be supported by a "reasoned explanation**." (*In re Nuvasive*, Inc., 842 F.3d 1376,1383 (2016) citing *Lee*, 277 F.3d at 1342, 1345. Further, "references to 'common sense' ... cannot be used as a wholesale substitute for reasoned analysis and evidentiary support...." (*Id.*, citing *Arendi S.A.R.L. v. Apple Inc.*, 832 F.3d 1355, 1362 (Fed. Cir. 2016)).

In presenting the *prima facie* case, it is well established that deriving the claimed technology through *ex post* hindsight reasoning is impermissible. In particular, "**[i]t is**

impermissible to use the claimed invention as an instruction manual or 'template' to piece together the teachings of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 972 F.2d 1260, 1266, (Fed. Cir. 1992); *ATD Corp. v. Lydall, Inc.*, 159 F.3d 534, 546 (Fed. Cir. 1998), emphasis added. ("obviousness cannot be based on the hindsight combination of components selectively culled from the prior art to fit the parameters of the patented invention".)

Consequently, an examiner absolutely must avoid using hindsight, and the legal conclusion must be reached on the basis of the facts gleaned from the prior art. See MPEP § 2142. At the outset, hindsight must not be used to define the problem that the invention solves. *Mintz v. Dietz & Watson, Inc.*, 679 F.3d 1372 (Fed. Cir. 2012). Then, hindsight must not be used to gather or suggest the elements of the invention. See *In re Ochiai*, 71 F.3d at 1572 (Fed. Cir. 1995). Finally, "both the suggestion and the expectation of success must be founded in the prior art, not in the Appellant's disclosure." *In re Dow Chemical Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988).

E. Detailed argument

1) The Office has mischaracterized the teachings of Grow, and all rejections rely on conclusions drawn from the mischaracterization

The Office highlights the following passage from the Abstract of Grow:

> 30. Grow et al., abstract, teach the preparation of a fecal sample for immunoassay, which includes determining if human hemoglobin is present. As disclosed therein:
>
> A method for preparing a fecal sample for immunoassay testing comprising the steps of dispersing **a sample of from 1 to less than 10 wt. % of a stool sample** in an aqueous fecal test solution formulated with one or more preservatives, analyte stabilizing agents and endogenous interference reducing agents. The fecal solids are then permitted to settle to form a liquid phase substantially free from fecal solids, and the clarified liquid phase is removed to provide a test sample free from fecal solids. **The fecal test solutions contain suitable stool stabilizers such as buffers** and antimicrobial agents, analyte protecting agents such as proteolytic, reductive or oxidative enzyme inhibitors, endogenous assay interfering enzyme inhibitors such as a reducing agent, and non-specific binding inhibitors such as animal proteins. **The stool sample should be fresh or be fresh frozen** and thawed immediately before dispersion in the buffer solution. The sample is suitable for any solid-phase immunoassay determination of a fecal sample analyte. A method for determining analyte in the stool sample comprises conjugating anti-analyte antibody adhered to an insoluble support with analyte in the clarified sample, and determining the presence and extent of such conjugation. For determining human hemoglobin in a sample of human stool, the aqueous fecal test solution preferably contains a proteinase inhibitor, formaldehyde and an animal albumin. (Emphasis added)

(Office Action, ¶30).

Citing this passage, the Office then asserts that "The fact that only 1 to less than 10% of the sample is being used is deemed to meet a limitation of claim 82, step b)." (see Office Action at ¶31).

The Office has mischaracterized what Grow plainly says. Grow does not say 1 to less than 10% [of the sample] but recites "1 to less than 10 **wt.** %", which has an entirely different meaning. "**Wt.%**" refers not to a fraction of the sample. It is a standard abbreviation for "weight percent," or the weight or mass fraction of one component, especially a solid (*e.g.*, stool sample) relative to the total weight or mass of a solution or suspension (*e.g.*, Grow's aqueous fecal test solution with dispersed stool sample in it). Read accurately and in its entirety, the Abstract of Grow describes a preparation of fecal material dispersed in an "aqueous fecal test solution" in which the stool sample is "from 1 to less than 10 wt. %" of the total composition, with the balance of the weight (from 99% to greater than 90%) being the weight of the aqueous fecal test

solution. The term "wt. %" is used consistently throughout Grow and has nothing to do with a subportion of a fecal sample.

Grow says nothing about using *only a portion* of a fecal sample, nor is partitioning of a fecal sample inherent in, or required by, the method of Grow. For example, taking the weight of 100% of *any* size of fecal sample as a first part, the skilled artisan readily understands adding more than 9 parts of aqueous fecal test solution to arrive at a mixture that is less than 10 wt.% stool sample in the resulting dispersed sample preparation, with no need to remove any portion of the fecal sample.

Grow thus fails to provide the limitation of claim 82, step b).

The Office also points to the Abstract of Grow above as teaching that "the sample can be frozen," and alleges that this meets the limitation of claim 83. (Office Action, ¶31.)

However, claim 83 depends from claim 82, and the freezing step recited in claim 83 applies only to the "remaining portion" of the fecal sample that is produced as set forth in claim 82, step b), *i.e.*, by first removing the "removed portion" of the fecal sample. Grow's mention that a "stool sample should be fresh or frozen" makes no mention of partitioning a fecal sample, and does not teach either producing a "remaining portion" from a fecal sample as set forth in claim 82, step b), or freezing the remaining portion after removal of the removed portion.

Grow does not and cannot meet the limitations of claim 83 because Grow fails to meet the limitations of claim 82.

In rejecting each of claims 82-84 and 87-100, the Office relies *entirely* on this mischaracterization of Grow for a teaching of the limitation of claim 82, step b), as described above. Each and every one of Appellant's pending claims includes this element, by dependence from or reference to claim 82. Thus, the rejection of each of the claims relies on the Office's erroneous reading of Grow. The Office has erred in making the rejections for at least this reason.

2) The combination of references fails to teach all the features of the invention as claimed

Each and every pending claim includes the limitation of claim 82, step b), as described above, by dependence from or reference to claim 82. Neither Appellant's alleged admissions, nor

any of Yee, Shuber, or Goggins teach the limitation of claim 82, step b), so do not cure the deficiency.

Further, each and every claim includes the pair of separately stabilized portions produced by the method recited in claim 82: a removed portion and a remaining portion from the same fecal sample.

Appellant's alleged admissions do not include these features, nor do the secondary references enumerated in the Office Action teach them. The secondary references are directed to immunoassay analysis of fecal samples for occult blood/hemoglobin (Yee and Grow) **or** for stabilizing fecal samples for nucleic acid analysis (Shuber). Neither the Appellant's alleged admissions nor secondary references Yee, Grow, or Shuber teach or suggest testing for both occult blood/hemoglobin and nucleic acid in the same fecal sample. Thus, it is not surprising that none of the references cited by the Office, taken alone or in any combination, teach or suggest producing a removed portion and a remaining portion from the same fecal sample, and separately stabilizing the pair of portions, as set for in claim 82 and the remaining claims. Goggins, cited as teaching, *inter alia*, methylation of the FOXE1 gene (a gene listed in Appellant's claim 99) in association with pancreatic carcinoma (Office Action, ¶¶46-50), does not cure the deficiency.

The combination of references thus fails to teach all the elements of claims 82-84 and 87-100, and does not establish *prima facie* obviousness of these claims. The Office has therefore further erred in making the rejections for at least this reason.

3) The Office relied on conclusory statements and has failed to provide articulated reasoning with proper rational underpinning to support the legal conclusion of obviousness, and has relied on improper hindsight reconstruction to arrive at Appellant's claims

As discussed above, "[r]ejections on obviousness grounds **cannot be sustained by mere conclusory statements**; instead, **there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness**." *KSR Int'l v. Teleflex Inc.*, 127 S. Ct. 1727, 1741 (2007). Further, "**[i]t is impermissible to use the claimed invention as an instruction manual or 'template' to piece together the teachings of the prior art so that the claimed invention is rendered obvious.**" *In re Fritch*, 972 F.2d 1260, 1266, (Fed. Cir.

1992); *ATD Corp. v. Lydall, Inc.*, 159 F.3d 534, 546 (Fed. Cir. 1998).

Regarding claims 82-84 and 87-100, in making the rejections of these claims, the Office states that "In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to analyze fecal samples for both blood (hemoglobin) as well as for gene/sequence of interest. (Office Action, ¶36).

This statement is entirely unsupported by evidence or argument. There is no "above showing" in the Office Action that contains any suggestion to analyze fecal samples for both blood (hemoglobin) as well as for gene/sequence of interest.

The summary of teachings in part C of this paper, above, lists the teachings and limitations to which the Office points in characterizing Appellant's alleged admissions, and in characterizing Yee, Grow, Shuber, and Goggins. As discussed above, none of the references cited by the Office, taken alone or in any combination, teach or suggest producing a removed portion and a remaining portion from the same fecal sample, and separately stabilizing the pair of portions, and the Office has failed to provide any proper rationale for modifying the teachings of the references to arrive at Appellant's claims.

The Office has enumerated the teachings of Yee and Grow with respect to testing samples for blood, and has not identified, for example, any deficiencies of blood/hemoglobin testing of fecal samples as taught by Grow and Yee that are curable by the addition of Shuber's DNA testing. Conversely, the Office has pointed to Shuber as teaching the benefits of treating a stool sample with a nucleic acid stabilization solution (Office Action, ¶¶33-34), but has not identified any deficiencies of DNA testing of fecal samples as taught by Shuber that are curable by addition of Yee and Grow's blood/hemoglobin testing. The Office has pointed to nothing in these references that suggests that any benefit or improvement that reasonably could be expected by subjecting a single fecal sample to both types of tests.

Office further states that "It would also have bene [sic] obvious to collect separate portion of a sample and to treat each in a manner that would either preserve the protein target(s) (Grow et al.) or the nucleic acid target(s) (Shuber et al.)." (Office Action, ¶36).

This statement is also unsupported by evidence or reasoned argument. As discussed above, the Office has provided no evidence suggesting that combining a test for a blood protein target and a test for a nucleic acid target from a single fecal sample would or might provide any kind of benefit or improvement. Having failed to provide any reason for the skilled artisan to subject a single fecal sample to both types of tests, the Office has also failed to provide any rationale for the invention in the embodiment as claimed, *i.e.*, for producing a removed portion and a remaining portion from the same fecal sample, and separately stabilizing the pair of portions, as set forth in claim 82.

The Office's sole argument in support for combining the references consists of the following statement:

> 37. In view of the intense interest in detection of colorectal carcinoma in an individual, said ordinary artisan would have been amply motivated. And in view of the well-developed state of the art, and the fact that one would be using the disclosed methods for the same purpose, said ordinary artisan would have had a most reasonable expectation of success.

(Office Action, ¶37)

In pointing to "the intense interest in detection of colorectal carcinoma in an individual" as a basis for asserting that the skilled person would be motivated to combine the references and to modify and piece together their teachings to arrive at Appellant's claimed embodiment, the Office has failed to articulate precisely what the skilled person would be seeking to achieve by combining the references, *e.g.*, how making the combination would serve to address "the intense interest in detection of colorectal carcinoma in an individual."

As noted above, the Office has not identified a single deficiency in any of the cited art nor a single benefit conferred by combining the teachings to motivate the skilled person to combine the references. Nothing in these references suggests performing both testing methods on a single fecal specimen might offer any particular improvement or benefit over the use of one testing method alone.

Further, as discussed above, *each and every pending claim* includes the pair of portions of a fecal sample: a removed portion and a remaining portion from the same fecal sample,

separately stabilized. The pair of portions is produced by the method recited in claim 82. Even if some advantage of using both the blood and DNA testing on feces from a subject is postulated, the Office has provided no reasoning or rational as to why the skilled person would be motivated to apply the different tests from the cited art on the same fecal sample.

Rather, the skilled person, considering the cited art, is left to guess at whether expending the time and effort to conduct both blood/hemoglobin testing and nucleic acid testing on a single fecal sample *might* produce some beneficial result, rather than merely producing redundant diagnostic information.

Although the Office asserts that the skilled person would have been amply motivated to make the combination by "intense interest in detection of colorectal carcinoma in an individual," the Office has not shown why, based on the cited art and Appellant's alleged admission, a skilled person would believe that testing a fecal sample using both blood/hemoglobin testing *and* DNA testing would be expected to offer any benefit with respect to detection of colorectal carcinoma at all, much less a benefit of sufficient importance or magnitude to offset the increase in costs and labor of running both of these different tests on each sample, as compared to running a single type of test on the sample. (The Office asserts at ¶41 of the Office Action that Appellant has provided no evidence to support that the amount of work would be increased by performing both tests, but it is objectively factual that performing one test is less work than performing that test, plus performing a second, different test on a separate portion of the sample.)

As discussed above, "It is impermissible to use the claimed invention as an instruction manual or 'template' to piece together the teachings of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 972 F.2d 1260, 1266, (Fed. Cir. 1992); *ATD Corp. v. Lydall, Inc.*, 159 F.3d 534, 546 (Fed. Cir. 1998). "[H]indsight" is inferred when the specific understanding or principal within the knowledge of one of ordinary skill in the art leading to the modification of the prior art in order to arrive at appellant's claimed invention has not been explained." *In re Rouffet*, 149 F.3d 1350, 1358 (Fed. Cir. 1998).

Appellant's Specification teaches the benefits of testing a fecal sample for both blood proteins (referenced as fecal immunoassay testing, or "FIT" testing) and DNA marker modification. See, *e.g.*, Example 1 starting on page 43. For example, for fecal samples in sample set A, Table 3 on page 49 show sensitivities of 52% to 70% for samples tested for

methylation of the combination of NDRG4, FOXE1, and PHACTR marker genes, and Table 4 on the same page shows sensitivity of 78% by FIT testing the same set A of fecal samples. Table 6, bridging pages 50-51, shows the results of the combining these test methods, with sensitivities raging from 91% to 96% with the combined testing on sample set A.

In the context of the technology disclosed by Appellant, there is the requirement for appropriate sample processing. To meet this requirement, Appellant's technology provides a method of sample processing comprising removing a portion of a collected fecal sample and adding the removed portion of the sample to a buffer which prevents denaturation or degradation of blood proteins found in the fecal sample, and separately stabilizing the remaining sample, *e.g.*, to preserve DNA in the remaining portion. One particular benefit is that a fecal sample may be collected and partitioned into separately stabilized portions at home by the subject, *e.g.*, prior to delivery to a laboratory. (See, *e.g.*, the Specification at page 28, lines 1-7 of the second full paragraph.)

Given the absence of any suggestion of partitioning fecal samples in the art cited by the Office, it can only be concluded that the Office has done precisely what is forbidden, and has used the disclosure of Appellant's Specification and claims as a guide to selectively cull from the prior art elements that fit the parameters of the claims, using Appellant's disclosure as a template to piece together the embodiments as claimed. The Office has erred in making the rejections for at least this reason.

Regarding Claim 99, the Office cites Appellant's alleged admissions, Yee, Grow, and Shuber as applied to claims 82-84, 87-98, and 100, further in view of Goggins.

The Office admits that the combination of Appellant's alleged admissions, Yee, Grow, Shuber does not teach the genes listed in claim 99. (Office Action, ¶45). The Office points to Goggins as teaching, *inter alia,* the FOXE1 mutation in association with pancreatic carcinoma (Office Action, ¶47).

The Office asserts that "it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the teachings of applicant, Yee et al., Grow et al., and Shuber et al., whereby the gene to be evaluated in terms of methylation was FOXEl as it had already been associated with cancer." (Office Action, ¶50).

However, the *pancreatic* cancer of Goggins is the wrong cancer to support the Office's argument for the alleged obviousness of combining Appellant's alleged admissions, Yee, Grow, Shuber. The Office fails to explain how Goggins's disclosure of the FOXE1 marker in association with *pancreatic* cancer might point the skilled person to process a fecal sample in the manner of the instant claims. Notably, Goggins does not disclose fecal samples as a source of cells to be tested. (see Goggins at ¶0042, as cited in Office Action at ¶48). Nothing in Goggins suggests any utility in testing fecal samples, much less testing the same fecal sample using both blood/hemoglobin testing *and* nucleic acid testing for the FOXE1 gene for *pancreatic* cancer (or any other cancer).

Still further, Goggins' teaching of FOXE1 as a useful marker for *pancreatic* carcinoma does not in any way suggest a utility in line with the "intense interest in detection of *colorectal* carcinoma in an individual," which is the sole motivation offered by the Office for combining the Appellant's alleged admissions, Yee, Grow, and Shuber. To the contrary, if, as the Office has argued, detection of the methylation status of the FOXE1 gene in a sample collected from a colon is indicative of *pancreatic* carcinoma, then the skilled person considering the teachings of Goggins *would exclude* FOXE1 for detecting *colorectal* carcinoma by analysis of such samples, as Goggins teaches that detecting methylation of FOXE1 in a sample is indicative of a different type of carcinoma. The Office has improperly relied on Appellant's own Specification for the teaching to test FOXE1 DNA in a stabilized remaining portion of a fecal sample in association with detecting *colorectal* cancer, and further in association with testing a removed portion of the same fecal sample for occult blood/hemoglobin.

The Office asserts that Goggins' teaches that "the neoplasm" can be that which occurs in the colon (Office Action, ¶49, citing Goggins at ¶0079), seemingly suggesting that Goggins links methylation of FOXE1 with colorectal carcinoma. The Office again misrepresents the teachings of the cited reference. Paragraph 0079 of Goggins provided a *general* definition of "a neoplasm" as creating an unstructured mass or tumor, and that such a neoplasm "may be a head, neck, lung, esophageal, stomach, small bowel, colon, bladder, kidney, or cervical neoplasm":

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

(Goggins, ¶0079, emphasis added)

Read in context, it is clear that Goggins is teaching that neoplasms *in general* may occur in any of these sites. This paragraph in no way suggests any link between colorectal neoplasm and methylation of the FOXE1 gene.

Lastly, as discussed above, Goggins fails to cure the deficiencies of the Office's arguments regarding Appellant's alleged admissions, Yee, Grow, and Shuber as applied to claim 82, as nothing in Goggins teaches or suggests testing for both occult blood/hemoglobin and methylation of FOXE1 in the same fecal sample, nor a method of partitioning and separately stabilizing the portions of the fecal sample, as set forth in claim 82.

CONCLUSION

In view of the points discussed above, it is clear that the Office has erred and has failed to establish *prima facie* obviousness of claims 82-84 and 87-100 over Appellant's alleged admissions in view of Yee, Grow, and Shuber, with or without Goggins, and that these rejections should be reversed.

Respectfully submitted,

Dated: March 9, 2021 /Mary Ann D. Brow/

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V. CLAIMS APPENDIX

1-81. (canceled)

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

a) collecting a fecal sample from a human subject;

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

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

d) stabilizing the remaining portion of the fecal sample.

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

i) adding a stabilizing buffer to said remaining sample;

ii) freezing said remaining sample.

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

85-86. (canceled)

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

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

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

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

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

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

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

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

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

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

95. The method of claim 87, further comprising

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

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

96. The method of claim 95, wherein said epigenetic modification comprises aberrant

methylation.

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

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

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

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

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	1252	1	640	640
Miscellaneous:				
		Total in USD ($)		**640**

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Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
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First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	173254
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Appeal Brief Filed	2021-03-09_35239-304_AppellantsBriefFINAL.pdf	307666 107a1dbe096526d3b353107cea3abbff685b7762	no	24
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30860 24b421fbfa51b77ab86e836a67fb2314d1691910	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	338526		

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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

173254 7590 06/28/2021

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

EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
06/28/2021	ELECTRONIC

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

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

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

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

Office Action Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Bradley L Sisson	Art Unit 1634	AIA (FITF) Status No

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

Period for Reply

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

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

Status

1)☑ Responsive to communication(s) filed on 09 March 2021.

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

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

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

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

Disposition of Claims*

5) ☑ Claim(s) 82-84 and 87-100 is/are pending in the application.

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 82-84 and 87-100 is/are rejected.

8) ☑ Claim(s) 82-83 is/are objected to.

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

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

Application Papers

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

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

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

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

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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

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

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

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

Attachment(s)

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

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

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

4) ☐ Other: _____.

DETAILED ACTION

Prosecution Reopened after Appeal Brief

1. In view of the appeal brief filed on 09 March 2021, PROSECUTION IS HEREBY REOPENED. New ground of rejection is set forth below.

2. To avoid abandonment of the application, appellant must exercise one of the following two options:

(1) file a reply under 37 CFR 1.111 (if this Office action is non-final) or a reply under 37 CFR 1.113 (if this Office action is final); or,

(2) initiate a new appeal by filing a notice of appeal under 37 CFR 41.31 followed by an appeal brief under 37 CFR 41.37. The previously paid notice of appeal fee and appeal brief fee can be applied to the new appeal. If, however, the appeal fees set forth in 37 CFR 41.20 have been increased since they were previously paid, then appellant must pay the difference between the increased fees and the amount previously paid.

3. A Supervisory Patent Examiner (SPE) has approved of reopening prosecution by signing below:

/DAVE T NGUYEN/
Supervisory Patent Examiner, Art Unit 1634

Notice of Pre-AIA or AIA Status

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

Claim Objections

5. Claims 82 and 83 are objected to because of the following informalities: In claim 82, there is no conjunctive (and) or disjunctive (or) following step c), and before step d). Similarly,

claim 83 lacks a conjunctive or disjunctive following step i), but before step ii). Appropriate correction is required.

Claim Rejections - 35 USC § 103

6. 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.

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

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

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

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

9. It is further noted that prior art is not limited to the four corners of the documentary prior art being applied. Prior art includes both the specialized understanding of one of ordinary skill in the art, and the common understanding of the layman. It includes "background knowledge possessed by a person having ordinary skill in the art. . . [A] court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *KSR* at 1396.

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

Admissions as Prior Art.

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

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

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

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

Holding and Rationale

13. Claims 82-83, and 87 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41) in view of US 2008/0124714 A1 (Shuber et al) and US 2006/0216830 A1 (Kikuiri).

14. Kutzner et al., disclose method for the detection of colorectal tumors using a combination of tests, one which looks for a component of blood, and one which is based on nucleic acid analysis, e.g., PCR.

15. A seen therein, the faecal occult blood test was "a standard guaiac test", which is not an immunological test, deemed deficient, and does not seek to determine the amount of hemoglobin present. Kutzner et al., have not been found to teach freezing a portion of the sample (claim 83) or assaying for aberrant methylation, including the aspect of modifying DNA with a bisulfite ion which results in unmethylated cytosine being converted to uracil (claims 95-98 and 100).

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

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

17. Shuber et al., in Example 2, teach the benefits of treating a stool sample with a nucleic acid stabilization solution. Addition of stabilizer and the freezing step are also disclosed on page 32. And in Example 3, "The Effect of Stabilization Buffers on DNA Integrity and Multiple Mutation Assays", Shuber et al., teach performing nucleic acid analysis on frozen samples where

a stabilizing agent had been used. Such a showing is deemed to meet a limitation of claims 82 and 83.

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

19. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the methods of Kutzner et al., whereby one was detecting for colorectal cancer by using multiple distinct portions from each fecal sample, as shown above. Such methods had been developed to the point that one would have been motivated to obtain a high degree of sensitivity by directing a parallel nucleic acid assay for known, informative genes, whereby one was using different portions of a faecal sample for the immunoassay and the nucleic acid-based assay. See Kultzer et al. It would have also been obvious to said ordinary artisan to have employed means of stabilizing the sample portions for to do so would have a direct an immediate effect of being able to accurately and reproducibly determine the level of hemoglobin present, as well as preserve any nucleic acid present in the cells, and therein enhancing the ability to obtain accurate results, as suggested by Shuber.

20. Claims 87-94 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41) in view of US 2008/0124714 A1 (Shuber et al), as applied to claim 82, and further in view of US 2006/0216830 A1 (Kikuiri) and Yeh (US 2008/0227298 A1).

21. The rejection of claim 82 as being obvious over Kutzner in view of Shuber is applied here as indicated above. Kutzer in view of Shuber does not teach limitations such as delivering

fecal samples and separate portions to a lab, hemoglobin detection with various concentrations that are known in the prior art as indicative of either a threshold level or upper level therefrom.

22. However, Kikuiri, at paragraph [0051], teaches that container with a fecal specimen can be transported/delivered to a medical facility for testing. As stated therein:

> [0051] The feces sampling container 1 containing the fecal suspension 17a is directly transported to a medical facility or the like, and subjected to a quantitative analysis of a specific component included in the fecal suspension 17a. With reference to FIGS. 6 to 8, the structure of an analyzer will be described, by taking an analyzer for measuring a hemoglobin concentration in the fecal suspension 17a as an example. (Emphasis added)

23. As evidenced above, the sample can be tested for concentration of hemoglobin. The above showing is deemed to meet a limitation of claim 84.

24. While Kikuiri teaches detecting hemoglobin in a fecal sample, Kikuiri has not been found to teach the concentration of hemoglobin that can be detected.

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

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

> Fecal occult blood tests fall primarily into two categories, tests based on the use of chromogenic chemical reagents such as gum guaiac and immunochemical tests. The chemically based guaiac methods determine the presence of occult blood by the detection of the perioxidase activity of the hemoglobin in the blood present in the faecal sample. They require catalysis of peroxide into oxygen and water, and the subsequent oxidation of a colorless dye (most often into a colored form). However, peroxidase activity is also found in meats and vegetables. In order to produce accurate results, these tests require restriction of the intake of certain foods, drugs, vitamins, and other substances prior to and during the sample collection period. The sensitivity of the most commonly used guaiac FOBT (Hemoccult) is approximately 50%. Despite a specificity of 98%, the positive predictive value for FOBT is low. Methods of detecting occult blood based on porphyrin (heme and protpoporphyrin IX) analysis or immunologic tests using anti-hemoglobin antibodies improve on these results. **Immunochemical tests (FIT or**

> **iFOBT) that use anti- hemoglobin antibodies specific for human blood in extracts from stool do not require dietary restrictions;** however, they are more complicated and more expensive than peroxidase-based tests. (Emphasis added).

27. It would have also been obvious to an ordinary artisan to have employed distinct portions from a subject's fecal sample in testing lab, as exemplified by Kikuiri, to detect multiple signals indicative of colorectal cancer or tumor, e.g., an inclusion of detecting occult blood (hemoglobin) at various desired contractions based on known threshold levels. The ordinary artisan would have been motivated perform multiple testing on different targets present in a fecal sample in order to enhance various degrees of accurate diagnosis of colorectal cancer, as taught and suggested by the combined cited references, especially hemoglobin detections with established thresholds are well established in the prior art as exemplified by Yee.

28. Claims 87, 95-100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41) in view of US 2008/0124714 A1 (Shuber et al), as applied to claim 87, and further in view of either Kim et al. (BMB reports, Volume 41, Issue 10, Pages 685-692, September 2008) or WO 2008/084219 A1 (Van England et al.).

29. The rejection of claim 87 as being obvious over Kutzner in view of Shuber is applied here as indicated above. Kutzer in view of Shuber does not teach limitations such as specific non-invasive molecular biomarker detection with various epigenetic biomarkers. e.g., hypermethylation level.

30. However, Kim teaches colorectal cancer epigenetic biomarkers are used for the early detection along of colorectal cancer with fecal-occult blood test (FOB). See page 686, Table 1, particularly column 1 bridging column 2 of page 689.

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

> Van England relates to detection of colorectal cancer based upon determining methylation of a number of different genes, including panels of genes. The elected marker NDRG4 is also taught in Van England et al. (claims 1 and 2).

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

> **Determination of the methylation status may be achieved through any suitable means. Suitable examples include bisulphite genomic sequencing and/or by methylation specific PCR. Various techniques for assessing methylation status are known in the art and can be used in conjunction with the present invention**: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR(McMS-PCR), MLPA with or without bisulphite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulphite conversion-specific methylation-specific PCR (BS-MSP)(Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence diferences in PCR products of sodium bisulphite - treated DNA), methylation-sensitive single-nucleotide primer extension conformation(MS-SNuPE), methylation- sensitive single-strand conformation analysis (MS-SSCA), Melting curve combined bisulphite restriction analysis (McCOBRA)(Akey et al., 2002), PyroMethA, HeavyMethyl (Cottrell et al. 2004), MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles (QAMA), enzymatic regional methylation assay (ERMA), QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques for DNA methylation analysis is provided in Nucleic acids research, 1998, Vol. 26, No. 10, 2255-2264, Nature Reviews, 2003, Vol.3, 253-266; Oral

> Oncology, 2006, Vol. 42, 5- 13, which references are incorporated herein in their entirety. (Emphasis added)

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

> In the MSP approach, DNA may be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulphite treatment (**Herman et al., 1996; and WO 97/46705**). For example, bisulphite ions modify non-methylated cytosine bases, changing them to uracil bases. Uracil bases hybridize to adenine bases under hybridization conditions. (Emphasis added)

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

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

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

36. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the methods of Kutzner et al. in view of Shuber, whereby one was detecting hemoglobin via an immunoassay for as shown above. Such methods had been developed to the point that one would have been motivated to obtain a high degree of sensitivity by directing a parallel nucleic acid assay for known, informative genes, such as the

elected NDG4, TFPI2, PHACTR3, SFRP2 and SOX17, whereby one was using different portions of a faecal sample for the immunoassay and the nucleic acid-based assay, e.g., hypermethylation detection assays. It would have also been obvious to said ordinary artisan to have employed means of stabilizing the sample portions for to do so would have a direct an immediate effect of being able to accurately and reproducibly determine the level of hemoglobin present, as well as preserve any nucleic acid present in the cells, and therein enhancing the ability to obtain accurate results.

37. In view of the teaching of the combined cited art such as Kutzner and Kim, which teaches a desire for accurate detection of early colorectal cancer with usages of various known molecular DNA biomarkers in addition with the FOBT, one of ordinary skilled artisans would have been motivated have employed admittedly known methods so to detect not only occult blood (hemoglobin) but known and useful gene targets, and to also determine whether the target sequences such as NDRG4 also demonstrate hypermethylation, be it in a gene of interest or a promoter of same.

38. In view of the well-developed state of the art and significant interest in being able to detect cancer at an early stage via both immunochemistry and nucleic acid analysis, said ordinary artisan would have been both well motivated and would have had a most reasonable expectation of success.

Conclusion

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

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

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

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

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

/Bradley L. Sisson/

Primary Examiner, Art Unit 1634

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

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20060216830-A1	09-2006	Kikuiri; Hideki	B01L3/5082	436/165
*	B	US-20080227208-A1	09-2008	Yee et al.	G01N33/72	436/66
	C					
	D					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
*	N	WO-2004083399-A2	09-2004	WO	Goggins et al.	C12Q1/6886
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kutzner et al., "Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", Cancer Letters", 229 (2005), pp. 33-41. (Year: 2005)
	V	Kim et al., "Noninvasive molecular biomarkers for the detection of colorectal cancer", BMB reports, Volume 41, Issue 10, pp 685-692, September 2008
	W	
	X	

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

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

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

CLAIMS

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

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021				
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021				
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CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021				
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
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CPC - Searched*		
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US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

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Search Notes		
Search Notes	**Date**	**Examiner**
EAST (see transcript for databases and search terms)	06/23/2019	B. L. S.
Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.
EAST (see transcript for databases and search terms)	01/14, 15/2020	B. L. S.
STN (see transcript for databases and search terms)	01/14/2020	B. L. S.
EAST (updated)	08/04/2020	B. L. S.
EAST	06/16/2021	B. L. S.

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

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Non-invasive detection of colorectal tumours by the combined application of molecular diagnosis and the faecal occult blood test

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Abstract

The treatment of early-stage tumours decreases the overall mortality of colorectal tumour patients. In this retrospective study we determined the sensitivity and the specificity of the faecal occult blood test (FOBT) and the molecular diagnosis (MD). We analysed 57 stool samples from patients with colorectal carcinomas for the presence of occult blood using a standard FOBT and for alterations in the three different tumour relevant markers APC, BAT26 and L-DNA. Stool samples from 44 control donors were analysed to determine the specificity of the applied methods. Twenty-nine (51%; 95% confidence interval (CI): 38–63%) stool samples of the cancer patients gave positive FOBT results. Thirty-seven (65%; CI: 52–76%) samples showed alterations in at least one DNA marker. Sixteen (28%) samples were positive only in the FOBT, and 24 (42%) samples showed a positive result exclusively in MD. The combined application of both methods resulted in a sensitivity of 93% (CI: 83–97%) and an overall specificity of 89% (CI: 76–95%). The combined application of FOBT and MD resulted in an overall sensitivity, which could not be achieved by any of the methods alone and which is in the range of invasive diagnostic methods.
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Keywords: Non-invasive colorectal cancer diagnosis; Faecal DNA analysis; Molecular diagnosis; Faecal occult blood test

1. Introduction

The diagnosis and the therapy of early-stage tumours have the potential to decrease morbidity and mortality of colorectal cancer patients. Invasive methods like sigmoidoscopy, colonoscopy and

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roentgenography are valuable tools for the specific and sensitive detection and the exact localization of a colorectal tumour (reviewed in [1]). These techniques are unsuitable for broad screening programs mainly because of their low general acceptance [2]. The most common screening method is the faecal occult blood test (FOBT). Randomised trials revealed the clinical effectiveness in reduction of mortality through the implementation of the FOBT [3–5].

Sidransky et al. introduced the analysis of faecal DNA for tumour specific alterations as a novel non-invasive diagnostic strategy [6]. Until now several studies have proven the usefulness and the feasibility of faecal DNA analysis (reviewed in [7]). Recent multi-target studies achieved sensitivities between 62 and 91% and specificities up to 98%, respectively [8–10]. A few studies compared the outcomes of the molecular diagnosis (MD), which is the detection of tumour relevant alterations in faecal DNA, with these of the FOBT. The finding that 9 out of 11 asymptomatic adenoma patients were identified by the analysis of five different DNA markers, whereas none was detected by FOBT, indicated a higher diagnostic sensitivity of the MD [9]. The data of a large prospective study, which is close to completion, will allow assessing the two methods and their combined application (annual meetings of the American College of Gastroenterology (ACG), Baltimore 2003 and of the American Gastroenterology Association (AGA), New Orleans 2004). We intended to judge in this retrospective study whether the analysis of three faecal DNA markers has the potential to complement or even to replace the FOBT and whether the combined application of the two methods might increase the overall diagnostic reliability.

2. Materials and methods

2.1. Study population and sample collection

All patients and control donors provided their informed oral and written consent to provide samples. This study was approved by the responsible ethical committees at the Klinik für Chirurgie Marienhospital (Herne) and at the Berufsgenossenschaftliche Kliniken Bergmannsheil (Bochum). None of the patients and none of the control donors had familial adenomatous polyposis coli or hereditary non-polyposis colorectal cancer. Patients were diagnosed with colorectal tumours at the Berufsgenossenschaftliche Kliniken Bergmannsheil, the Marienhospital Herne or the Katholisches Krankenhaus Dortmund-West between 1998 and 2003. All tumours were diagnosed and located by imaging methods like colonoscopy and roentgenography. At least seven days after colonoscopy and between 2 and 4 days before surgical removal of the tumour 2 samples were taken from regular stools, immediately frozen at −20 °C and transferred to −80 °C within 24 h. The surgically removed tumours were classified according to the AJCC/TNM system (American Joint Committee on Cancer (AJCC) stages I through IV; Table 1) [11]. Because of personnel alternations in the clinical departments in the course of the study, we could not reproduce the staging information for 12 tumours, the age and the gender of 8 patients and the location for 3 tumours at the time of the analysis of the faecal samples. Control stool samples were from 24 female and 20 male donors, who volunteered to participate. The age group of control donors was 24–78 years (mean age 49). Control donors did not show any symptoms or signs of colorectal diseases as determined in personal interviews.

2.2. FOBT

Two stool samples from each cancer patient and one sample from each control donor were analysed for the presence of occult blood by a standard guaiac test using commercial reagents and standard protocols (Care Diagnostica). Each FOBT was performed twice. A blue colour reaction within 120 s after the addition of the hydrogen peroxide solution was rated as a positive result. In case the double FOBT analysis of one stool sample or of two stool samples from one patient gave discrepant results, the sample was rated positive.

2.3. MD

Faecal DNA from all samples was purified using a method, which was optimised for faecal specimen [12]. An essential step of this method is the matrix-based elimination of PCR inhibiting and DNA damaging compounds (Stool Mini Kit, Qiagen).

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Table 1
Patient and tumor data of all samples and results obtained by the analysis of stool samples from cancer patients by FOBT and by MD

Patient	Gender/age	AJCC/TNM	FOBT	MD positive markers
79	M/70	n.a.*	neg	neg
80	M/58	II/T3N0MxG2	pos	APC
83	M/67	II/T3N0MxG2	neg	APC, L-DNA
84	M/85	II/T3N0MxG2	neg	BAT26
87	n.a.	n.a.	neg	APC
88	F/76	II/T3N0MxG2	pos	neg
89	n.a.	n.a.	neg	L-DNA
90	F/62	I/T2N0MxG2	neg	L-DNA
92	F/53	n.a.	pos	neg
93	n.a.	n.a.	pos	APC
95	F/68	I/T2N0MxG2	pos	APC
96	n.a.	n.a.	neg	APC, L-DNA
121	M/51	I/T2N0MxG2	neg	L-DNA
136	M/61	I/T2N0MxG2	neg	APC
138	M/65	I/T2N0MxG2	pos	neg
139	M/74	II/T3N0MxG2	neg	neg
140	F/73	II/T3N0MxG2	pos	neg
141	n.a.	n.a.	pos	APC
172	F/75	II/T3N0MxG2	neg	APC
173	M/73	II/T3N0MxG2	neg	APC
174	F/79	IV/T3N2M1hepG2	pos	APC, L-DNA
175	F/75	II/T3N0MxG3	pos	neg
176	M/63	IV/T3N1M1hepG3	neg	APC
179	M/68	III/T3N1MxG2	neg	APC
180	F/65	II/T4N0MxG2	neg	APC
181	F/92	III/T4N1M0G2	pos	neg
182	M/74	I/T2N0MxG2	neg	APC
183	M/60	II/T3N0MxG2	neg	L-DNA
184	M/65	IV/T3N2M1hepG2	neg	L-DNA
185	M/75	I/T2N0MxG2	pos	BAT26
186	M/47	III/T3N1MxG2	neg	L-DNA
187	M/73	II/T3N0MxG2	pos	neg
188	M/65	IV/T3N1M1hepG2	neg	neg
189	M/76	III/T3N2MxG2	neg	APC, L-DNA
190	M/69	I/T2N0MxG2	pos	BAT26, L-DNA
191	M/76	III/T3N1MxG2	pos	L-DNA
192	M/89	II/T3N0MxG2	pos	L-DNA
193	F/64	I/T2N0MxG2	neg	BAT26, L-DNA
251	M/55	IV/T3N2M1G2	pos	L-DNA
252	M/74	II/T3N0MxG2	pos	neg
253	F/83	III/T3N1MxG2	neg	L-DNA
254	M/66	II/T4N0MxG2	pos	BAT26, L-DNA
255	M/62	III/T3N2MxG2	pos	neg
257	n.a.	n.a.	neg	APC
274	F/70	I/T2N0MxG3	pos	neg
275	n.a.	n.a.	pos	APC, L-DNA
276	n.a.	n.a.	neg	L-DNA
278	M/86	n.a.	neg	L-DNA
283	M/82	III/T3N1MxG2	pos	APC, L-DNA
284	F/72	n.a.	neg	L-DNA
285	M/81	II/T3NxMxG2	neg	neg
286	M/64	IV/T3NxM1hepG2	pos	neg

(*continued on next page*)

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Table 1 (*continued*)

Patient	Gender/age	AJCC/TNM	FOBT	MD positive markers
287	M/92	III/T3N1MxG3	pos	neg
289	M/71	II/T4NxMxG3	pos	neg
290	M/66	IV/T4N0M1	pos	neg
292	M/80	I/T1N0MxG2R0	pos	neg
294	F/83	III/T3N2MxG2	pos	neg

n.a., data not available

The quality of the purified DNA was validated by agarose gel electrophoresis. One tenth of the DNA obtained from one stool sample was used in a capturing step to enrich the human DNA from the DNA of non-human origin [13]. The overall yield of the capturing could be increased by the restriction digestion of the DNA with the enzyme DraI. To fish the APC gene out of the pool of genomic DNA, 10 pmol biotin labelled primer APC1 (5′-TTAAAA-TATGCCACAGATATTCCT TCA TCACAGAAA-CAGT-3′) was added to 100 μl DraI digested stool DNA. After denaturing and re-annealing 10 μl streptavidin coupled magnetic beads (Dynal) were added and the mixture was incubated over night at room temperature. After washing, the beads coupled DNA was completely used as template in a first PCR. The APC gene region from codon 1069 through codon 1984 including the mutation cluster region was analysed by PCR and by PTT of two overlapping fragments. The detailed experimental conditions of the following PTT have been published [13] (Hauss and Müller, The protein truncation test in mutation detection and molecular diagnosis, in: Methods in Molecular Biology, edited by Guido Grandi, The Humana Press, in press). Briefly, the primers of the first PCR coded the HA-tag, and the primers of the second PCR coded the Kozak motif and the T7 promoter sequence, which are necessary for the following PTT. The first PCR was carried out at the following conditions: 95 °C (5 min)//[94 °C (30 s)/56 °C (30 s)/72 °C (90 s)] 20×//[94 °C (30 s)/58 °C (30 s)/72 °C (90 s)] 20×//72 °C (10 min) and under the use of the following primers: APC fragment I: 5′-CGCCATGTACCCCTACGACGTGCCCGACTAC GCCTTCCAACCACATTTTGGACAGCAG-3′ and 5′-CCGTCATTTTTCTGCCTCTTTCTCTTGGTT-3′; APC fragment II: 5′-CGCCATGTACCCCTAC-GACGTGCCCGACTACGCCGATGTGGAATTAA-GAATAATGCCT-3′ and 5′-CCGTCATTTTCTT-TATTGTTGTTTTCTTGGTC-3′. One fifth of the volume of the first PCR was used in a second PCR at the conditions: 95 °C (5 min)//[94 °C (30 s)/60 °C (30 s)/72 °C (90 s)] 40×//72 °C (10 min) and using the following primers, which anneal within the products of the first PCR: APC fragment I: 5′-ATCCTAATACGACTCACTATAGGGAGCCAC-CATGTACCCCTACGACGTG-3′ and 5′-CCGTCA-TTTTTCTGCCTCTTTCTCTTGGTT-3′; APC fragment II: 5′-ATCCTAATACGACTCACTATAGGG-AGCCACCATGTACCCCTACGACGTG-3′ and 5′-CCGTCATTTTCTTTATTGTTGTTTTCTTGGTC-3′. Three of the control samples gave no reproducible results in the PCR reactions. All other faecal DNA samples from control donors and all samples from cancer patients gave robust PCR products. DNA purified from two stool samples from each cancer patient and from one sample from each control donor was analysed for the presence of alterations in 3 different markers. Each positive result was confirmed in a repeated analysis. All samples showing signals, which were different to the wild-type controls, were further examined. For this the PCR fragments were cloned in a T/A cloning vector and the cloned gene fragments were sequenced by conventional methods. The analyses of all positive samples were repeated by starting with the purified DNA in order to limit the number of false positive results. The analysis of alterations in the microsatellite marker BAT26 and the detection of the relative amount of non-apoptotic L-DNA were performed according to published protocols [9,14]. All results are based on the analysis of at least two independently amplified PCR products. The primer sequences for L-DNA analysis were kindly provided by A.P. Shuber and D.A. Ahlquist. In case of discrepant MD results between the two different samples from one patient, DNA was extracted from a new faecal aliquot of the same patient and the analysis was repeated.

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Table 2
Localization of tumours relative to the splenic flexure of the colon and number of the corresponding stool samples diagnosed as positive by FOBT or by MD

Site	*N*	FOBT	MD	Combined use
Proximal	27	16	14	25
Distal	27	11	21	26
n.a.	3	2	2	2
Total	57	29	37	53

N, number of samples; n.a., at the time of analysis the information of the tumour localization was not available.

2.4. Statistical evaluation

Ninety-five percent confidence intervals were determined based on the exact binomial distribution. The Fisher exact test was used for comparison of the test results between patients with different tumour stages. To compare the single application of FOBT or MD with the combined application of both methods the McNemar's test was used.

3. Results

The aim of this study was to determine the sensitivity and the specificity of the two non-invasive diagnostic methods, FOBT and MD, which is the analysis of faecal DNA for alterations in the three different markers APC, BAT26 and L-DNA. We analysed stool samples from 57 colorectal cancer patients and from 44 control donors. Twenty-seven tumours were located proximal and 27 tumours were located distal to the splenic flexure of the colon (Table 2). Twenty-eight patients had tumours, which did not spread to proximal or distant lymph nodes (stage I or II) at the time of staging diagnosis. Lymphatic spread was identified in 17 patients (stage III or IV) (Tables 1 and 3).

Twenty-nine stool samples (51%; 95% confidence interval (CI): 38–63%) showed a positive FOBT test (FOBT pos). Thirty-seven faecal DNA samples (65%; CI: 52–76%) were MD positive (MD pos) demonstrating an alteration in at least one of the three tested DNA markers (Tables 1 and 3). The MD positives include nineteen samples (33%) with truncated APC protein fragments in the PTT analysis (Fig. 1). We sequenced these positive samples and found mutations, which result in stop codons between codons 1254 and 1472 (Table 4). The sizes of the predicted translation products of PCR fragments carrying these stop codons correlate with the approximate sizes of the translation products, which were detected in the PTT (Fig. 1). The number of APC mutations detected in the samples was in the range of the numbers

Table 3
Summarized results of the analysis of the stool samples from cancer patients and sensitivity of MD and FOBT

AJCC stage	n.a.	I	II	III	IV	Total
N	12	11	17	10	7	57
Mean age (range)	70.3 (53–86)	67.2 (51–80)	72.7 (47–92)	76.1 (47–92)	65.3 (55–79)	70.9 (47–92)
Gender (male/female)	2/2	7/4	12/5	7/3	6/1	34/15
n.a.	8	0	0	0	0	8
FOBT pos	4	6	9	6	4	29 (51%; 38–63%)
MD pos	10	8	9	6	4	37 (65%; 52–76%)
APC	6	3	5	3	2	19 (33%)
BAT26	0	3	2	0	0	5 (9%)
L-DNA	6	4	4	5	3	22 (39%)
MD pos or FOBT pos	11	11	15	10	6	53 (93%; 83–97%)
MD(APC or L-DNA) pos or FOBT pos	11	11	14	10	6	52 (91%; 81–96%)
MD pos and FOBT neg	7	5	6	4	2	24 (42%; 30–55%)
FOBT pos and MD neg	1	3	6	4	2	16 (28%; 18–41%)

The results, the relative sensitivities and the corresponding 95% confidence intervals are given for the FOBT, the MD and the combined application of the two methods. A sample was rated MD positive (MD pos), when one molecular marker was tested positive in the PTT, in the BAT26 or in the L-DNA analysis. n.a., AJCC staging of the corresponding tumours or age and gender were not available at the time of analysis.

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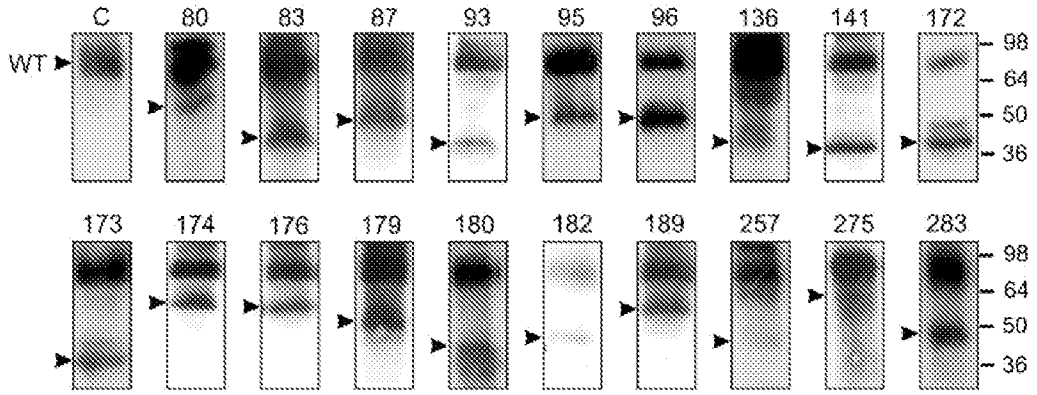


Fig. 1. Positive PTT results of the APC gene analysis. Arrowheads point to bands, which represent the wild-type full-length protein (WT) or smaller proteins, which are truncated due to mutations. On the right side the sizes of marker fragments are indicated in kilodaltons. C, non-mutated control.

described in other studies [9,15]. Ahlquist et al. (2000) detected APC mutations in 24% faecal DNA samples of cancer patients. Traverso et al. (2002) found APC mutations in 63% DNA samples of patients with T3N0M0 tumours. We detected BAT26 patterns different to the wild-type control in 5 (9%) samples (Fig. 2). Twenty-two (39%) samples showed high levels of L-DNA amplification products (Fig. 3). Thirty-five stool samples (61% of all samples or 95% of MD positive samples) showed alterations in the APC gene or in the relative L-DNA level. This high number reflects the high proportion of colorectal tumours, which harbour an APC gene mutation and a dysfunction in the regulation of apoptosis (Tables 1 and 3).

Forty-two percent of the samples (CI: 30–55%) were positive in at least one of the MD markers, but gave negative FOBT results. Twenty-eight percent of the samples (CI: 18–41%) were positive exclusively in the FOBT, but not in the molecular test. When the results of both methods were combined, 53 (93%; CI: 83–97%) samples were positive in FOBT or in molecular testing. The combined application of the two methods together leads to a significantly higher sensitivity than the application of any of the methods alone ($P < 0.01$). When FOBT was combined with a limited MD, which is the analysis of the two molecular markers APC and L-DNA, a detection rate of 91% (CI: 81–96%) was achieved. The sensitivity of this combined application is higher than the sensitivity of the analysis by limited MD or by FOBT alone ($P < 0.01$).

In one control sample we found occult blood by FOBT resulting in an overall FOBT specificity of 98% (CI: 88–100%) (Table 5). In another control sample we detected a truncated APC protein fragment by PTT. The following sequence analysis revealed a C to T transition at nucleotide position 4117 leading to

Table 4
Sequence alterations in APC detected by sequencing of MD positive samples

Sample	Mutation at sequence position	Resulting stop at codon
80	C4278del	1472
83	C4117T	1367
87	G4153T	1379
93	G4075T	1353
95	C4366T	1450
96	C4366T	1450
136	G3943T	1309
141	A3835T	1273
172	G3943T	1309
173	C3777del	1254
174	4409–4412del	1472
176	A4330del	1472
179	G4152^G4153insA	1414
180	C4117T	1367
182	C4117T	1367
189	T4310^G4311insGCCACCAA	1472
257	C4117T	1367
275	A4382del	1472
283	C4055A	1346

Nucleotide changes, the nucleotide sequence positions and the resulting stop codons are listed for the detected APC mutations. All faecal DNA samples were screened by PTT for alterations in APC. Positive samples were sequenced.

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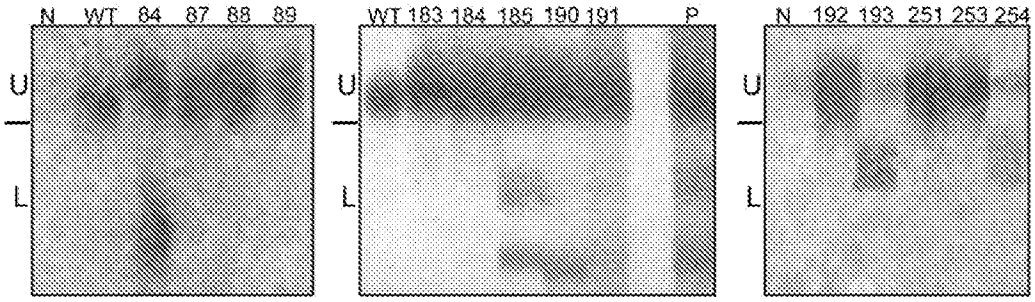


Fig. 2. Polyacrylamide gels for the analysis of alterations in the BAT26 microsatellite patterns. All BAT26 positive samples and 9 representing negative samples are shown. The upper (U) region of the gel contains PCR products representing the wild-type full-length products. Bands in the lower (L) regions of the gels indicate deletions in the BAT26 microsatellite. N, negative control without any DNA template; WT, wild-type control from normal tissue; P, positive control from tumour tissue containing the mutant BAT26.

a stop signal in the corresponding codon 1367 (not shown). Both control donors showed no signs of malignancies or polyps according to colonoscopy analysis. Three samples from the control group did not give reproducible PCR results. In the final statistical calculation of the overall specificity these control samples were rated positive (Table 5). Thus the specificity of MD was 91% (CI: 79–96%), and the overall specificity of the combined application of FOBT and MD was 89% (CI: 76–95%).

4. Discussion

Tumour specific mutations in faecal DNA are supposed to be sensitive and specific markers for the non-invasive diagnosis of colorectal cancer (reviewed in [7,16]). In this study the sensitivity of the retrospective diagnosis of colorectal carcinoma by the analysis of the three different molecular markers APC, BAT26 and L-DNA was 65%. The sensitivities described by others for the analysis of five different markers reach from 64 to 90% [9,10]. The differences between the sensitivities might be due to variations in sample quality, in the applied analytical methods and in the laboratory-specific handling of the samples. The high potential of the MD became obvious, when we compared the MD outcomes with those of the most common non-invasive screening test, the FOBT. The MD showed a 14% higher detection rate than the FOBT, and 42% of all samples were negative in the FOBT, but still detectable by MD. Nevertheless, our data also confirm the value of the FOBT. Transferring the data to a putative random non-invasive screen 28 out of 100 cancer patients would be detected only by FOBT but not by MD. The sensitivity of MD can be even further increased up to 93% by the additional application of FOBT. While the combination of the FOBT and MD leads to a significant increase in sensitivity, it leads only to a marginal decrease in specificity (Table 5).

There was no significant correlation of the FOBT or MD results with the gender, the age or the tumour stages. This finding is in line with the results of others [9]. Furthermore, we did not find a significant correlation of the positive markers with the location

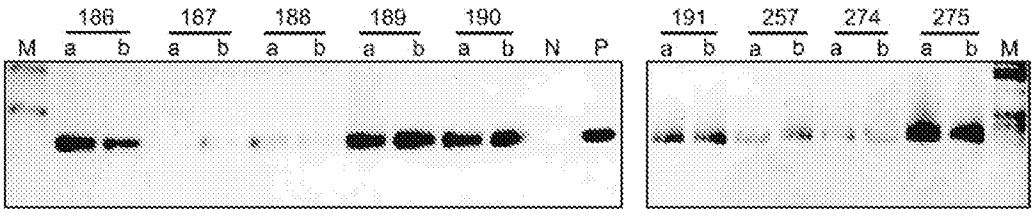


Fig. 3. Agarose gels for the analysis of the amplification of L-DNA. The two gels show the analysis of PCR of KRAS-L-DNA from 9 representing stool DNA samples, which were amplified in duplicates (a, b). M, Marker; N, negative control without any DNA; P, positive control.

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Table 5
The numbers of positive samples and Summary of results

	Cancer patients	Control donors	Sensitivity (%)	Specificity (%)
FOBT pos	29	1	51 (38–63)	98 (88–100)
MD pos	37	4	65 (52–76)	91 (79–96)
MD pos or FOBT pos	53	5	93 (83–97)	89 (76–95)
MD(APC or L-DNA) pos or FOBT pos	52	5	91 (81–96)	89 (76–95)

The relative sensitivities, specificities and the corresponding 95% confidence intervals of the MD, the FOBT and of the combined application of the two methods are given. The MD positive samples of the control donors include three samples, which could not be analysed.

of the tumours (Table 2). The findings of others, who described indications for a correlation between tumour location and BAT26 and L-DNA markers, have to be confirmed by the analysis of a high number of samples [9].

In order to determine the specificity we analysed the samples of randomly chosen control donors, who showed no obvious symptoms of colorectal diseases. This strategy might have led to an overestimated specificity in comparison to an authentic screening, which includes both non-symptomatic and symptomatic cancer patients and healthy donors. Because the analysed MD markers APC and BAT26 correlate directly to tumour development and do not necessarily correlate with the onset of symptoms, the analysis of these two markers should not distort the overall specificity. We are still aware that the FOBT outcomes might have led to the overestimation of the overall specificity. A subsequent study, which will include samples from non-symptomatic as well as samples from symptomatic individuals, will clarify this aspect.

This is one of the first studies, which evaluate and directly compare the potential of the two non-invasive methods MD and FOBT in the diagnosis of colorectal carcinomas. The combined application of the two methods together led to a significantly higher sensitivity than the application of any of the methods alone. Regarding the low cost and the little technical expense of the FOBT, we suggest that MD and FOBT should complement rather than replace each other. The sensitivity of the combined application of MD and FOBT is in the range reported for invasive methods. This leads to the question whether both methods should be used together in broad diagnostic screening programs. We are aware that our retrospective data do not allow any conclusion on the potential of this approach in diagnostic screening. Prospective comparative studies, which include a very high number of individuals, have to prove the value of the combined application of MD and FOBT. Preliminary results of a large study were presented in abstract form (ACG and AGA annual meetings). The final results of this study will allow further conclusions about the effectiveness of the two methods and their combined application.

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Mini Review

Noninvasive molecular biomarkers for the detection of colorectal cancer

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

Introduction

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

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

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

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

Fecal markers

Fecal hemoglobin

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

Genes and epigenetic markers

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

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

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

Table 1. Noninvasive molecular biomarkers for the detection of CRC

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

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

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

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

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

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

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

Serum or blood markers

CEA

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

CA 19-9

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

Tissue inhibitor of metalloproteinase type 1

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

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

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

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

Nicotinamide N-methyltransferase and proteasome activator complex subunit 3

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

Collapsin response mediator protein-2

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

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

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

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

α-Defensins

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

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Macrophage migration inhibitory factor

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

Macrophage-colony stimulating factor

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

Prolactin

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

M2-pyruvate kinase

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

Methylated septin-9 DNA

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

Five gene markers in whole blood

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

Other markers

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

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

Conclusion and perspectives

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

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

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

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	53	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:50
S2	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:51
S3	9	(hemoglobin haemoglobin) with concentration same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:18
S4	260	DNA same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:35
S5	2	"20080227208"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:34
S6	1	"20080227208" and "10 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:35
S7	11	(hemoglobin haemoglobin) with "5 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:38
S8	0	NDGR4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S9	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR;	OR	OFF	2020/01/14 13:50

			FPRS; EPO; JPO; DERWENT; IBM_TDB			
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S11	24	"2006113770"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:02
S12	4	"2006113770" and NDRG4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:11
S13	6	NDRG4 same (epigenetic methylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S14	6	NDRG4 same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S15	0	"N-myc downstream regulated gene 4" same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S16	0	"N-myc downstream regulated gene 4" and (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S17	0	"N-myc downstream regulated gene 4" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:25
S18	1	"20060019256" and uptg	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:55

S19	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 15:35
S20	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:07
S21	3	fecal same (antibody antibodies immunoassay) same (DNA RNA polynucleotide "nucleic acid" gene) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:26
S22	2	(cancer carcinoma) same (PHACTR3 NDRG4 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFRP2 CDO1) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:40
S23	1	"200483399"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:42
S24	0	"200483399A2"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:43
S25	0	"200483399A2"	EPO	OR	OFF	2020/01/15 11:11
S26	0	"200483399"	EPO	OR	OFF	2020/01/15 11:11
S27	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/08/04 18:14
S28	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/08/04 19:10
S29	15	hemoglobin with (stool fecal) with concentration and @pd<"20090203"	USPAT	OR	OFF	2021/06/15 10:13

S30	32	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and (antibody antibodies immunoassay immunological) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 11:33
S31	16	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same (antibody antibodies immunoassay immunological) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 11:33
S32	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same (antibody antibodies immunoassay immunological) and "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 11:37
S33	1,048	(freeze freezing frozen) same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:05
S34	399	(freeze freezing frozen) with (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:05
S35	236	(freeze freezing frozen) with (stool fecal faecal) with (sample fraction portion historical) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:07
S36	14	(freeze freezing frozen) with (stool fecal faecal) with (fraction portion historical) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:07
S37	4	(immunoassay elisa) with (stool fecal faecal) with (fresh "non-frozen") and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:31
S38	1	"7832219".FMID.	US-PGPUB; USPAT; FPRS; JPO	OR	OFF	2021/06/16 12:32
S39	4	"7832219"	USPAT	OR	ON	2021/06/16 12:33
S40	2	(freeze freezing frozen) with (stool fecal faecal) with (fraction portion	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2021/06/16 13:30

		historical) same buffer and @pd<"20090203"	DERWENT; IBM_TDB			
S41	5	(freeze freezing frozen) same (stool fecal faecal) with (fraction portion historical) same buffer and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 13:33
S42	230	"5741650"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 13:38
S43	114	"5741650"	USPAT	OR	ON	2021/06/16 13:38
S44	63	"5741650" and frozen	USPAT	OR	ON	2021/06/16 13:40
S45	65	"5741650" and (freeze freezing frozen)	USPAT	OR	ON	2021/06/16 13:41
S46	3	(feces fecal faecal faeces) with buffer with (freeze freezing frozen) and @pd<"20090203"	USPAT	OR	ON	2021/06/16 13:51

6/16/2021 4:59:26 PM
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Application	Document	Mailroom Date	Attorney Docket No.
15634607	CTNF	06/28/2021	EXCTD-35239.304
	892	06/28/2021	EXCTD-35239.304

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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

AMENDMENT AND RESPONSE TO NON-FINAL OFFICE ACTION MAILED 06/28/2021

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

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

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

Dated: November 29, 2021 By: /Mary Ann D. Brow/
Mary Ann D. Brow, Reg. No. 42,363

This paper is responsive to the Office Action mailed 06/28/2021, with response due by 09/28/2021. A two-month extension of time, to Monday, 11/29/2021, the first business day following Sunday, 11/28/2021, is requested. Applicant respectfully requests reconsideration in view of the amendments and remarks hereinbelow.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: **EXCTD-35239.304**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

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

Remarks begin on Page 5.

A**MENDMENTS TO THE CLAIMS:**

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

1-81. (canceled)

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

a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject by defecation directly into a sealable collection vessel;

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

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

d) stabilizing the remaining portion of the fecal sample by adding a buffer that maintains DNA integrity in a fecal sample to the remaining portion in the sealable collection vessel.

83. (canceled)

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

85-86. (canceled)

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

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

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

B) testing the removed portion of said fecal sample for ~~the presence of blood~~ a concentration of hemoglobin present in said removed portion,

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

D) analyzing said DNA for the presence of DNA from the human subject having an epigenetic modification.

88. (canceled)

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

90. (currently amended) The method of claim ~~88~~87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml.

91. (currently amended) The method of claim ~~88~~87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml.

92. (currently amended) The method of claim ~~88~~87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml.

93. (currently amended) The method of claim ~~88~~87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 50 ng/ml.

94. (currently amended) The method of claim ~~88~~87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml.

95. (canceled)

96. (currently amended) The method of claim ~~95~~87, wherein said epigenetic modification comprises aberrant methylation.

97. (currently amended) The method of claim ~~95~~87, wherein said aberrant methylation comprises hypermethylation.

98. (currently amended) The method of claim ~~95~~87, wherein said DNA from ~~a~~ the human subject comprises a gene and/or a promoter region of a gene.

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

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

REMARKS

In the Office Action mailed 06/28/2021 ("Office Action"), the Examiner made the following objections and rejections:

I. Claims 82 and 83 are objected to because of the following informalities: In claim 82, there is no conjunctive (and) or disjunctive (or) following step c), and before step d). Claim 83 lacks a conjunctive or disjunctive following step i), but before step ii).

II. Claims 82-83, and 87 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41, hereinafter "Kutzner") in view of US 2008/0124714 Al ("Shuber") and US 2006/0216830 Al ("Kikuiri").

III. Claims 87-94 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Kutzner in view of Shuber as applied to claim 82, and further in view of Kikuiri and US 2008/0227298 Al (Yee).

IV. Claims 87, 95-100 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Kutzner in view of Shuber as applied to claim 87, and further in view of either Kim (BMB reports, Volume 41, Issue 10, Pages 685-692, September 2008; hereinafter "Kim") or WO 2008/084219 Al ("Van Engeland").

Claim amendments

For business reasons and without acquiescing to the Examiner's arguments, and reserving the right to prosecute the original or similar claims in one or more future applications, claim 82 is herein amended to recite a method of processing a freshly collected fecal sample without freezing, wherein the fecal sample is collected at home by a human subject by defecation directly into a sealable collection vessel. The removed portion is removed to a separate container and combined with a buffer and a buffer that maintains DNA integrity is added to the remaining portion in the sealable collection vessel. Support for the amendment is found in the application as originally filed, e.g., at page 28, second paragraph; the paragraph bridging pages 38-39; the

paragraph bridging pages 43-44.

Claim 83 is canceled without prejudice and claim 84 is amended for consistency with claim 82.

Claim 87 is amended to incorporate the elements of claims 88 and 95, which are cancelled in view of the amendment to claim 87.

Claims 90-94 and 96-98 are amended to depend from claim 87 instead of cancelled claims 88 and 95.

These amendments do not add new matter.

Objections

I. Claims 82 and 83 are objected to because of the following alleged informalities:

- In claim 82, there is no conjunctive (and) or disjunctive (or) following step c), and before step d). Claim 83 lacks a conjunctive or disjunctive following step i), but before step ii).

Claim 82 as herein amended recites "and" following step c) and before step d). Claim 83 is canceled in the present amendment, rendering the objection moot.

The claims are not obvious

II. Claims 82-83, and 87 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Kutzner in view of Shuber and Kikuiri. Applicant respectfully disagrees that the cited references render the claims *prima facie* obvious.

It is well-settled law that establishing *prima facie* obviousness requires *at least* a suggestion of all of the features in a claim. *See In re Wada and Murphy, citing CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) and *In re Royka*, 490 F.2d 981, 985 (CCPA 1974)). See also MPEP §2143. Further, a valid *prima facie* case requires the Office to present "articulated reasoning with some rational underpinning", *KSR*, 550 U.S. at 418 (citing *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)), that one of skill in the art would have been motivated to combine the elements to yield the claimed combination and that one would have done so having a reasonable expectation of successfully carrying out the claimed combination. *In*

re Vaeck, 947 F.2d 488, 493 (Fed. Cir. 1991).

As discussed above, the instant claims as amended are directed to a method of processing a freshly-collected fecal sample from a subject without freezing, wherein the sample is collected at home by the subject by defecation directly into a sealable collection vessel, wherein the sample is divided into portions that are separately stabilized, *e.g.*, for preserving different types of analytes in each portion. In particular, a removed portion in a separate container is combined with a buffer that stabilizes blood proteins and the remaining portion is separately treated in the original collection vessel with a buffer that maintains DNA integrity in the fecal sample. A fecal sample collected in the claimed manner is suitable for detecting blood proteins in one portion and DNA in the other portion of the same fecal sample.

The Examiner points to Kutzner as disclosing detection of colorectal tumors using a combination of tests, one which looks for a component of blood, and one which is based on nucleic acid analysis, e.g., PCR analysis. (Office Action, ¶14).

While not acquiescing to the Examiner's characterization of Kutzner, Applicant points out that Kutzner describes a retrospective study of mutations and blood in frozen patient samples (Kutzner, p34, col 2). Kutzner does not teach or suggest a method of processing a freshly-collected fecal sample without freezing using a method comprising collection of a fecal sample at home by a human subject by defecation directly into a sealable collection vessel, or wherein a portion of the fecal sample is removed to a separate container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample, as recited in instant claim 82. As acknowledged by the Examiner, Kutzner also does not suggest testing a fecal sample for a concentration of hemoglobin, much less testing a removed portion collected as described in the instant claims for a concentration of hemoglobin. The Examiner also acknowledges that Kutzner does not teach assaying DNA for aberrant methylation.

Further, Kutzner clearly states that their "retrospective data *do not allow any conclusion* on the potential of this approach [combined application of molecular diagnosis (MD) and fecal occult blood test (FOBT)] in diagnostic screening" and state that "Prospective comparative studies, which include a very high number of individuals, have to prove the value of the combined application of MD and FOBT." (Kutzner, p40, paragraph bridging columns 1-2,

emphasis added). Thus, Kutzner specifically states that it is not obvious to the authors of the reference, as skilled artisans, that combined testing of MD markers with FOBT will yield any particular result with respect to diagnostic screening. Kutzner provides no more than a guess that the approach *might be* successful, but does not provide a "reasonable expectation of success" required to establish *prima facie* obviousness.

Combination with Shuber and Kikuiri does not cure the deficiencies of Kutzner. While Shuber provides a number of methods of stabilizing nucleic acids, Shuber does not teach a method of processing a freshly-collected fecal sample without freezing using a method comprising collection of a fecal sample at home by a human subject by defecation directly into a sealable collection vessel, or wherein a portion of the fecal sample is removed to a separate container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample.

While Kikuiri discloses a device for collecting a sample of feces for hemoglobin testing, the device of Kikuiri is not configured to collect a fecal sample from a human subject by defecation directly into the container. See, for example, Fig. 4 of Kikuiri, illustrating specimen sampling member 20, "pinched with fingers" and showing specimen-sampling grooves 23*b* at the distal end. (see Kikuiri, ¶49). This illustration shows that the sample contemplated by Kikuiri is delivered into the container using a sample collecting member, rather than by defecation into the container. Further, Kikuiri make no suggestion of a buffer that "stabilizes blood proteins." Rather, Kikuiri also discloses only a "feces-dissolving liquid".

The combination of Kutzner, Shuber, and Kikuiri does not teach or suggest processing a freshly-collected fecal sample without freezing using a method comprising collection of a fecal sample at home by a human subject by defecation directly into a sealable collection vessel, or wherein a portion of the fecal sample is removed to a separate container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample.

While not acquiescing that the other requirements for establishing *prima facie* obviousness have been met, Applicant submits that, for the reasons set forth above, Kutzner in

view of Shuber and Kikuiri does not set forth all the elements of claims 82-83, and 87 and for at least this reason does not render these claims *prima facie* obvious. Applicant therefore respectfully requests that the rejection be withdrawn.

III. Claims 87-94 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Kutzner in view of Shuber as applied to claim 82, and further in view of Kikuiri and Yee.

The combination of Kutzner, Shuber, and Kikuiri as applied to claim 87 is discussed above. Claim 88 is canceled in the present amendment and remaining claims 89-94 depend from claim 87 and thereby comprise all the same elements of claim 87. As discussed in detail below, Yee does not cure the deficiencies of Kutzner, Shuber, and Kikuiri.

The Examiner admits that Kutzer in view of Shuber does not teach limitations such as delivering fecal samples and separate portions to a lab, or hemoglobin detection with various concentrations (Office Action, ¶21). The Examiner points to Kikuiri as teaching a container in which a fecal specimen can be transported. However, as discussed above, the device of Kikuiri is not configured to collect a fecal sample from a human subject by defecation directly into the container, or for transportation of a buffer-stabilized "remaining portion" to a medical facility for testing.

The Examiner further asserts that "it would have also been obvious to an ordinary artisan to have employed distinct portions from a subject's fecal sample in testing lab, as exemplified by Kikuiri, to detect multiple signals indicative of colorectal cancer or tumor, e.g., an inclusion of detecting occult blood (hemoglobin) at various desired contractions based on known threshold levels." (Office Action, ¶27).

Applicant disagrees with the Examiner's characterization of Kikuiri. While Kikuiri discloses a device for collecting a sample of feces for hemoglobin testing, Kikuiri makes no mention of what happens to any portion of the feces that is not collected into the disclosed device. Kikuiri makes no mention of employing distinct portion**s (plural)** from a subject's fecal sample in a testing lab, nor of "detecting **multiple signals** indicative of colorectal cancer or tumor," as alleged by the Examiner.

The Examiner further asserts that "The ordinary artisan would have been motivated

perform multiple testing on different targets present in a fecal sample in order to enhance various degrees of accurate diagnosis of colorectal cancer, as taught and suggested by the combined cited references" (Office Action, ¶27). However, this statement is not supported by the cited references or by any other evidence. As noted above, Kutzner clearly states that their "retrospective data *do not allow any conclusion* on the potential of this approach in diagnostic screening" and that prospective comparative studies that include very high number of individuals would be required to prove the value of the combined application of molecular testing and FOBT. (Kutzner, p40, paragraph bridging columns 1-2). Thus, it is not obvious even to the authors of the reference, as skilled artisans, that combined testing of molecular markers with FOBT will yield any particular result with respect to the accuracy of diagnostic screening for colorectal cancer. Kutzner provides no more than a guess that the approach *might be* successful. Kutzner's express statement that results presented "**do not allow any conclusion to be formed"** contradicts the rationale offered by Examiner, and shows that an expectation of enhanced accuracy of diagnosis of colorectal cancer could not be deemed a *reasonable* expectation from the teachings of these references.

Regarding a threshold value for hemoglobin, the Examiner points to Yee as setting a 10 ng/ml of hemoglobin in blood as a threshold value. However, without acquiescing to the Examiner's characterization of Yee, like Kikuiri, Yee makes no mention of analyzing multiple portions of a fecal specimen, nor of partitioning a fecal sample for combining different types of testing. Thus, while Yee may disclose a fecal test reciting a 10 ng/ml for hemoglobin, Yee does not cure the deficiencies of Kutzner, Shuber, and Kikuiri with respect to instant claims 87 and 89-94, as the combination of these references does not teach or suggest obtaining a pair of portions of a fecal sample from a human subject without freezing using a method comprising collection of the fecal sample at home by the human subject by defecation directly into a sealable collection vessel, wherein a portion of the fecal sample is removed to a separate container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample, nor of testing the removed portion of the fecal sample for a concentration of hemoglobin and testing DNA from the remaining portion of the same fecal sample for DNA from the human subject that has an epigenetic modification.

For the reasons set forth above, Applicant submits that the combination of Kutzner, Shuber, Kikuiri, and Yee does not establish *prima facie* obviousness of claims 87 and 89-94 as amended for at least the reason that the combination of references does not teach or suggest all the elements of the claims, and that the modifications urged by the Examiner are not supported by any reasonable expectation of success. Applicant therefore respectfully requests that the rejection be withdrawn.

IV. Claims 87, 95-100 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Kutzner in view of Shuber as applied to claim 87, and further in view of either Kim or Van Engeland.

The non-obviousness of claim 87 over Kutzner and Shuber as applied to claim 87 is discussed above and is applied here. Claim 95 is canceled in the present amendment and remaining claims 96-100 depend from claim 87 and thereby comprise all the same elements of claim 87.

As acknowledged by the Examiner in ¶29 of the Office Action, Kutzer in view of Shuber does not teach limitations such as specific non-invasive molecular biomarker detection with various epigenetic biomarkers. *e.g.,* hypermethylation level.

The Examiner points to Kim or Van Engeland as allegedly teaching colorectal cancer epigenetic markers. (Office Action, ¶¶30-31). However, neither Kim nor Van Engeland cure the deficiencies of Kutzner and Shuber discussed above. Further, Van Engeland is not prior art to the instant application.

Regarding Van Engeland, Applicant notes that the present application claims priority to U.S. Provisional Application No. 61/149,581, filed on February 3, 2009, which is less than one year from the publication date of the Van Engeland reference. Van Engeland therefore could be prior art to the present application only under pre-AIA 35 U.S.C. § 102(a).

Applicant has attached hereto an Inventor's Declaration Under 37 C.P.R. § 1.132 by inventor of the present invention Joost Louwagie ("Louwagie Decl."), which was originally filed with the Office in Appl. Ser. No., 13/147,570, to which the instant application claims priority as a continuation of U.S. Application No. 15/010,436, filed January 29, 2016, which is a continuation of U.S. Application No. 13/147,570. Inventor Louwagie states in ¶3 of the

Declaration that "In so far as the '219 Int'l Published App [WO 2008/084219] is asserted to disclose methods of determining a predisposition to, or the incidence of, colorectal cancer in a fecal sample from a human patient, comprising detecting methylation of one or more CpG dinucleotide motifs in the promoter of NDRG4 DNA contained within the fecal sample, these methods were invented by me and no other." Thus, the Louwagie Decl. established that the Van Engeland publication cited by the Examiner does not disclose invention "by another," as required under pre-AIA 35 U.S.C. § 102(a), and therefore does not qualify as prior art to the present application under § 102(a).

Examiner points to Kim as allegedly teaching "colorectal cancer epigenetic biomarkers are used for the early detection along of[sic] colorectal cancer with fecal-occult blood test (FOB)" pointing to page 686, Table 1, particularly column 1 bridging column 2 of page 689. (Office Action, ¶30). Applicant respectfully disagrees with the Examiner's characterization of Kim, as Kim makes no mention of testing epigenetic biomarkers along with FOB on the same fecal specimen. Rather, Kim is a review article that catalogues the "noninvasive molecular biomarkers that are currently in clinical settings and have the potential for use as CRC biomarkers" as of the time Kim was published in 2008 (Kim, Abstract). While Kim references the NDRG4 marker, nothing in Kim teaches or suggests combining detection of NDRG4 (or any other colorectal cancer epigenetic biomarkers) along with fecal-occult blood testing on separately stabilized portions of the same freshly collected fecal sample, as recited in Applicant's claims.

Thus, Kim does not cure the deficiencies of Kutzner and Shuber with respect to instant claims 87 and 96-100, as the combination of these references does not teach or suggest obtaining a pair of portions of a fecal sample from a human subject without freezing using a method comprising collection of the fecal sample at home by the human subject by defecation directly into a sealable collection vessel, wherein a portion of the fecal sample is removed to a separate container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample, nor testing the removed portion of the fecal sample for a concentration of hemoglobin and testing DNA from the remaining portion of the same fecal sample for DNA

from the human subject that has an epigenetic modification.

The Examiner further asserts that "In view of the teaching of the combined cited art such as Kutzner and Kim, which teaches a desire for accurate detection of early colorectal cancer with usages of various known molecular DNA biomarkers in addition with the FOBT, one of ordinary skilled artisans would have been motivated have[sic] employed admittedly known methods so to detect not only occult blood (hemoglobin) but known and useful gene targets, and to also determine whether the target sequences such as NDRG4 also demonstrate hypermethylation, be it in a gene of interest or a promoter of same" (Office Action, ¶37). However, teaching "a desire for accurate detection of early colorectal cancer" is not a teaching that the combination can be expected to successfully provide the desired result. As noted above, Kutzner clearly states that their "retrospective data *do not allow any conclusion* on the potential of this approach in diagnostic screening" and that prospective comparative studies that include very high number of individuals is required to prove the value of the combined application of molecular testing and FOBT. (Kutzner, p40, paragraph bridging columns 1-2). Thus, it is not obvious even to the authors of the reference, as skilled artisans, that combined testing of molecular markers with FOBT will yield any particular result with respect to the accuracy of diagnostic screening for colorectal cancer. Kutzner provides no more than a guess that the approach *might be* successful. Kutzner's express statement that results presented "**do not allow any conclusion to be formed"** contradicts the rationale offered by Examiner, and shows that an expectation of enhanced accuracy of diagnosis of colorectal cancer could not be deemed a *reasonable* expectation from the teachings of these references. It is, at best, a desired outcome.

For the reasons set forth above, Applicant submits that the combination of Kutzner, Shuber, and Kim does not establish *prima facie* obviousness of amended claim 87 and 96-100 for at least the reasons that the combination of references does not teach or suggest all the elements of the claims, and that the modifications urged by the Examiner are not suggested by the cited art or other evidence, nor are they supported by any reasonable expectation of success. Applicant therefore respectfully requests that the rejection be withdrawn.

Applicant's alleged admissions

In addition to the references discussed above, the Examiner characterized a number of

items from Applicant's Specification as applicant admissions. See, e.g., Office Action at ¶¶ 32, 32, 33, and 37. While the alleged applicant admissions are not enumerated or discussed here, the absence of discussion in this response is not to be taken as a concession that any information from Applicant's Specification is conceded by the Applicant to be prior art to the instant claims, nor that the Examiner's characterization of any such alleged admission is correct.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that all grounds for rejection have been addressed and Applicant's claims should be passed to allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicant encourages the Examiner to call the undersigned at (608) 662-1277.

Dated: November 29, 2021

/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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

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

Dear Commissioner:

I, Joost Louwagie, hereby declare and state that:

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

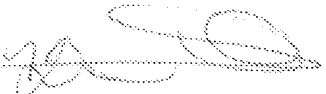
2. I am the sole inventor of the methods of determining a predisposition to, or the incidence of, colorectal cancer in a fecal sample from a human patient, comprising (a) detecting human blood in the fecal sample; and (b) detecting methylation of one or more CpG dinucleotide motifs in the promoter of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

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

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

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

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

By  Date: July 7, 2016

Joost Louwagie

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	1252	1	640	640
Miscellaneous:				
			Total in USD ($)	**640**

Electronic Acknowledgement Receipt

EFS ID:	44384222
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	173254
Filer:	Mary Ann Deese Brow/Stephanie Filandrinos
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	29-NOV-2021
Filing Date:	27-JUN-2017
Time Stamp:	16:53:26
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$640
RAM confirmation Number	E2021ASG53382438
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2021-11-29_35239-304_ROAb.pdf	1736148 dcda44c903a74cb12e7cc2b674b5c565e503cbf4	yes	16
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Claims		2	4	
	Applicant Arguments/Remarks Made in an Amendment		5	16	
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38400 e9f4697cde42c3633f1fcb9efed1a625d5659072	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1774548		

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/634,607	Filing Date 06/27/2017	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	11/29/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

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

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	SLIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/DEIDRE C KING-WOODWARD/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

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

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

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

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

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Joost		Louwagie	

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

City	Dornach	Country of Residence	CH

Mailing Address of Inventor:

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

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

Correspondence Information:

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

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

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

Application Information:

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

Patent Owner's Ex. 2003, Page 623
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

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

Filing By Reference:

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

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

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

Publication Information:

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

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

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

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

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

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

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

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

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

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

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

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

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

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

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

Authorization or Opt-Out of Authorization to Permit Access:

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

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

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

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

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

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

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

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

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

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

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

Applicant Information:

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

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

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

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

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name: ~~Exact Sciences Development Company, LLC~~ Exact Sciences Corporation

Mailing Address Information For Applicant:

Address 1	5505 Endeavor Lane		
Address 2			
City	Madison	**State/Province**	WI
Country	US	Postal Code	53719
Phone Number		Fax Number	
Email Address			

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

Assignee Information including Non-Applicant Assignee Information:

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

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

Assignee 1

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

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

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

Mailing Address Information For Assignee including Non-Applicant Assignee:

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

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

Signature:

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

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

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

Signature	/Mary Ann D. Brow/			Date (YYYY-MM-DD)	2019-05-23
First Name	Mary Ann D.	Last Name	Brow	Registration Number	42363

Additional Signature may be generated within this form by selecting the Add button.

Patent Owner's Ex. 2003, Page 628
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	EXCT-35239/US-4/CON
	Application Number	
Title of Invention	METHODS FOR DETECTING COLORECTAL CANCER	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Doc Code: R46C.REQ

Document Description: Request under 37 CFR 1.46(c) to correct, update or change the applicant.

PTO/AIA/41 (04-15)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

REQUEST TO CORRECT OR UPDATE THE NAME OF THE APPLICANT UNDER 37 CFR 1.46(c)(1), OR CHANGE THE APPLICANT UNDER 37 CFR 1.46(c)(2)

(FOR USE ONLY IN APPLICATIONS FILED ON OR AFTER SEPTEMBER 16, 2012)

Application Number	15/634,607
Filing Date	27-Jun-2017
First Named Inventor	JOOST
Art Unit	
Examiner Name	
Practitioner Docket Number	EXCTD-35239.304

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Applicant hereby **requests that the name of the applicant be corrected or updated under 37 CFR 1.46(c)(1), or that the applicant be changed under 37 CFR 1.46(c)(2),** in the above-identified application. **Requests under 37 CFR 1.46(c)(1) or (c)(2) cannot be submitted after payment of the issue fee or if the application has been patented.**

Please check the applicable box(es) below.

☒ 1. This request is to **correct or update the name of the applicant** (**under 37 CFR 1.46(c)(1)**) and includes:

☒ An application data sheet (ADS) in accordance with 37 CFR 1.76(c) with the corrected or updated information shown with markings (*e.g.*, underlining for insertions, strikethrough for deletions). A Corrected Web-based ADS may be used.

Note: Requests under 37 CFR 1.46(c)(1) may be filed to correct typographical errors in the name of the § 1.46 applicant, or for updating the name of the § 1.46 applicant (*i.e.*, where there is no change in the applicant itself but just in the applicant's name). See the Manual of Patent Examining Procedure (MPEP) section 605.01.

☐ 2. This request is to **change the applicant** (**under 37 CFR 1.46(c)(2)**) and includes:

☐ An application data sheet (ADS) in accordance with 37 CFR 1.76(c) that identifies the changes with proper markings (underlining for insertions and strikethrough for deletions). A Corrected Web-based ADS may be used.

☐ A Statement Under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent). See MPEP 325.

I am the

☐ applicant* ☒ attorney or agent of record Registration number 42363 ☐ attorney or agent acting under 37 CFR 1.34 Registration number _____

Signature /Mary Ann D. Brow/

Typed or printed name Mary Ann D. Brow

Date 2022-03-15

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. *Juristic entities must be represented by a patent practitioner (See 37 CFR 1.31, applicable to any paper filed on or after September 16, 2012 that is presented on behalf of a juristic entity, regardless of application filing date). Submit multiple forms if more than one signature is required, see below**.

☒ ** Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.36. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	45229344
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	173254
Filer:	Mary Ann Deese Brow/Jeremiah Gile
Filer Authorized By:	Mary Ann Deese Brow
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	15-MAR-2022
Filing Date:	27-JUN-2017
Time Stamp:	15:50:43
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	2022-03-15_35239-304_ADS-Marked-Up.pdf	141432 6a9ef364d279c6ed909c5225623d7c6404d9dfae	no	8

Warnings:

Information:					
This is not an USPTO supplied ADS fillable form					
2	Request under 37CFR1.46(c) to correct, update, change applicant	2022-03-15_35239-304_CorrApplForm.pdf	202369 37b1e4a5f7f1d0918817a66a5a5ec52e24964e64	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	343801	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874

173254 7590 03/25/2022

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
03/25/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DOCKETING@CASIMIRJONES.COM
Genomic_docketing@cardinal-ip.com

Office Action Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost	
	Examiner Bradley L Sisson	Art Unit 1634	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 11/29/2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 82,84,87,89-94 and 96-100 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 82,84,87,89-94 and 96-100 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Claim Interpretation

2. Attention is directed to MPEP 904.01 [R-08.2012].

> The breadth of the claims in the application should always be carefully noted; that is, the examiner should be fully aware of what the claims do not call for, as well as what they do require. During patent examination, the claims are given the broadest reasonable interpretation consistent with the specification. See *In re Morris*, 127 F.3d 1048, 44 USPQ2d 1023 (Fed. Cir. 1997). See MPEP § 2111 - § 2116.01 for case law pertinent to claim analysis.

3. It is noted with particularity that narrowing limitations found in the specification cannot be inferred in the claims where the elements not set forth in the claims are linchpin of patentability. *In re Philips Industries v. State Stove & Mfg. Co, Inc.*, 186 USPQ 458 (CA6 1975). While the claims are to be interpreted in light of the specification, it does not follow that limitations from the specification may be read into the claims. On the contrary, claims must be interpreted as broadly as their terms reasonably allow. See *Ex parte Oetiker,* 23 USPQ2d 1641 (BPAI, 1992). In added support of this position, attention is directed to MPEP 2111 [R-11.2013], where, citing *In re Prater*, 415 F.2d 1393, 1404-05, 162 USPQ 541, 550-51 (CCPA 1969), is stated:

> The court explained that "reading a claim in light of the specification, to thereby interpret limitations explicitly recited in the claim, is a quite different thing from 'reading limitations of the specification into a claim,' to thereby narrow the scope of the claim by implicitly adding disclosed limitations which have no express basis in the claim." The

court found that applicant was advocating the latter, i.e., the impermissible importation of subject matter from the specification into the claim.

4. Additionally, attention is directed to MPEP 2111.01 [R-07.2015], wherein is stated:

II. IT IS IMPROPER TO IMPORT CLAIM LIMITATIONS FROM THE SPECIFICATION

"Though understanding the claim language may be aided by explanations contained in the written description, it is important not to import into a claim limitations that are not part of the claim. For example, a particular embodiment appearing in the written description may not be read into a claim when the claim language is broader than the embodiment." *Superguide Corp. v. DirecTV Enterprises, Inc.*, 358 F.3d 870, 875, 69 USPQ2d 1865, 1868 (Fed. Cir. 2004).

5. Attention is also directed to MPEP 2111.02 II. As stated herein:

II. PREAMBLE STATEMENTS RECITING PURPOSE OR INTENDED USE
The claim preamble must be read in the context of the entire claim. The determination of whether preamble recitations are structural limitations or mere statements of purpose or use "can be resolved only on review of the entirety of the [record] to gain an understanding of what the inventors actually invented and intended to encompass by the claim." *Corning Glass Works,* 868 F.2d at 1257, 9 USPQ2d at 1966. **If the body of a claim fully and intrinsically sets forth all of the limitations of the claimed invention, and the preamble merely states, for example, the purpose or intended use of the invention, rather than any distinct definition of any of the claimed invention's limitations, then the preamble is not considered a limitation and is of no significance to claim construction.** *Pitney Bowes, Inc. v. Hewlett-Packard Co.,* 182 F.3d 1298, 1305, 51 USPQ2d 1161, 1165 (Fed. Cir. 1999). See also *Rowe v. Dror,* 112 F.3d 473, 478, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997) ("where a patentee defines a structurally complete invention in the claim body and uses the preamble only to state a purpose or intended use for the invention, the preamble is not a claim limitation")... (Emphasis added)

6. Attention is directed to MPEP 2111. As stated therein:

During patent examination, the pending claims must be "given their broadest reasonable interpretation consistent with the specification." The Federal Circuit's *en banc* decision in *Phillips v. AWH Corp.*, 415 F.3d 1303, 1316, 75 USPQ2d 1321, 1329 (Fed. Cir. 2005) expressly recognized that the USPTO employs the "**broadest reasonable interpretation**" standard:

> The Patent and Trademark Office ("PTO") determines the scope of claims in patent applications not solely on the basis of the claim language, but upon giving claims their broadest reasonable construction "in light of the specification as it would be interpreted by one of ordinary skill in the art." *In re Am. Acad. of Sci. Tech. Ctr.*, 367 F.3d 1359, 1364[, 70 USPQ2d 1827, 1830] (Fed. Cir. 2004). Indeed, the rules of the PTO require that application claims must "conform to the invention as set forth in the remainder of the specification and the terms and phrases used in the claims must find clear support or antecedent basis in the description so that the meaning of the terms in the claims may be ascertainable by reference to the description." 37 CFR 1.75(d)(1). (Emphasis added).

Claim Rejections - 35 USC § 103

7. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

8. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

9. Attention is directed to the decision in *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385 (U.S. 2007):

> When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense.

10. It is further noted that prior art is not limited to the four corners of the documentary prior art being applied. Prior art includes both the specialized understanding of one of ordinary skill in the art, and the common understanding of the layman. It includes "background knowledge possessed by a person having ordinary skill in the art. . . [A] court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *KSR* at 1396.

11. Suggestion, teaching or motivation does not have to be explicit and "may be found in any number of sources, including common knowledge, the prior art as a whole or the nature of the problem itself" *Pfizer, Inc. v. Apotex, Inc.* 480 F.3d 1348, 82 USPQ2d 1321 (Fed. Cir. 2007) citing *Dystar Textilfarben GMBH v. C. H. Patrick Co.*, 464 F.3d 1356 (Fed. Cir. 2006).

Admissions as Prior Art.

12. Attention is directed to MPEP 706.02 [R-07.2015]:

> **III. RELIANCE ON ADMITTED PRIOR ART IN SUPPORT OF REJECTION**
> A statement by an applicant in the specification or made during prosecution identifying the work of another as "prior art" is an admission which can be relied upon for both anticipation and obviousness determinations, regardless of whether the admitted prior art would otherwise qualify as prior art under the statutory categories of 35 U.S.C. 102. *Riverwood Int'l Corp. v. R.A. Jones & Co.*, 324 F.3d 1346, 1354, 66 USPQ2d 1331, 1337 (Fed. Cir. 2003); *Constant v. Advanced Micro-Devices Inc.*, 848 F.2d 1560, 1570, 7 USPQ2d 1057, 1063 (Fed. Cir. 1988). See MPEP § 2129 for discussion on admissions as prior art. Where the admitted prior art anticipates the claim but does not qualify as prior art under any of the paragraphs of 35 U.S.C. 102, the claim may be rejected as being anticipated by the admitted prior art without citing to 35 U.S.C. 102.

13. Attention is directed to MPEP 2129 [R-08.2012], Admissions as Prior Art, which states in part:

> **I. ADMISSIONS BY APPLICANT CONSTITUTE PRIOR ART**

> A statement by an applicant in the specification or made during prosecution identifying the work of another as "prior art" is an admission **>which can be relied upon for both anticipation and obviousness determinations, regardless of whether the admitted prior art would otherwise qualify as prior art under the statutory categories of 35 U.S.C. 102. *Riverwood Int'l Corp. v. R.A. Jones & Co.*, 324 F.3d 1346, 1354, 66 USPQ2d 1331, 1337 (Fed. Cir. 2003); *Constant v. Advanced Micro-Devices Inc.*, 848 F.2d 1560, 1570, 7 USPQ2d 1057, 1063 (Fed. Cir. 1988).

Holding and Rationale

14. Claims 82, 84, 87, 96, and 97 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41).

15. Wang et al., teach of a device that can be used for collecting a fecal sample, mixing said sample with a "preserving fluid", that the sample is not frozen (wet), yet is transported to a testing lab. As stated in the abstract:

> A device for quantitively **collecting, preserving, storing and mailing** a fresh and **wet** specimen of **fecal** or other biological matter for later analysis comprises a simple tubular vessel double sealed at one end by a machine manipulable plug having breakable hollow nib and cover, and that is engaged at the opposite end by a machine manipulable stopper from which a specimen carrying stick axially projects into the vessel through an internal sealable septum spanning a median section of the vessel. The cross-sectional profile of the stick is shaped to form a widened shoulder carrying a resilient washer. The shoulder matingly engages a correspondingly shaped passageway in the septum when the stopper engages the vessel which restricts the amount of specimen passing therethrough. The amount of specimen and **preserving fluid** are quantitatively balanced and remain so until part or all of the fluid is extracted for analysis either manually by breaking of the sealing nib or automatedly by machine removal of the plug or stopper. Collection by a patient or unskilled person is enhanced by providing the tubular vessel as part of a sample collection and return kit which further contains a disposable paper catch web which is made to be temporarily secured to a toilet droopingly spanning the seat to catch the feces for sampling. The kit also provides a sealable plastic bag and a crush resistant mailing

> carton. The above components of the kit are carried within a sterile bag until used. (Emphasis added)

16. Wang et al., at paragraph [0012], teaches:

> [0012] Collection by a patient or unskilled person is enhanced by providing the tubular vessel as part of a sampling kit which further contains a disposable paper catch web which is made to be temporarily secured to a toilet droopingly spanning the seat to catch the feces for sampling. The kit also provides a sealable plastic bag and a crush resistant mailing carton. The above components of the kit are carried within a sterile bag until used. (Emphasis added)

17. While Wang et al., teach collection of a sample, they have not been found to teach collecting a portion of a sample, or pairs of samples and sending these removed portion(s) to a lab for testing.

18. Liang et al., teach of a device and method for collecting and testing fecal samples. As disclosed in paragraph **[0112]**, one can collect multiple samples via a "sampling wand", which is then inserted into a test apparatus, which is subsequently transported to a testing laboratory. As disclosed therein:

> **[0112]** An apparatus as illustrated in FIGS. 3A-3 G or 5A-B, and containing appropriate assay buffer in the sample-receiving chamber, is provided to a user. The user collects his/her feces, for example on a flushable, paper sheet secured across the seat of a toilet. The user removes the cap and the sampling wand from the rapid test apparatus, and inserts the sampling wand into the fecal sample, for example multiple times in order to obtain sample from different portions of the sample. The sampling wand (member)/cap portion is then reinserted into the test apparatus, which causes insertion of the sampling wand into the **buffer** within the sample-receiving chamber of apparatus. The cap is tightened securely and the apparatus is gently shaken to solubilize the fecal sample to obtain a liquid suspension of buffer and the biological sample. The test apparatus is then transported, for example, via first class mail, to a laboratory. (Emphasis added)

19. It would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the method and device of Wang et al., so to include the device of Liang et al.,

for to do so would capitalize on the fecal sample being accessible on the covered toilet and collection of multiple samples that can be sent independently to the testing facility for analysis.

20. Liang et al., teach of various assays that can be performed. As seen in "Example 1" one can use the device for testing of fecal occult blood. However, Liang et al., has not been found to teach concentrations of hemoglobin that can be detected.

21. Neither Wang et al., nor Liang et al., have been found to teach testing DNA for epigenetic modifications.

22. Olek et al., disclose methods for diagnosing illnesses and predisposition to certain illnesses. As seen in paragraph [0003]:

> In contrast to classical mutations, **DNA methylation** involves a mechanism that describes a base substitution without modifying the coding function of a gene. This interplay between **epigenetic modification** and classical mutations plays an important role in tumorigenesis. For example, **focal hypermethylation and generalized genomic demethylation are features of many different tumor types**. It is assumed that tumorigenesis and tumor progression are caused, first of all, by **hypermethylation** of induced mutation events, and secondly, by the turning off of genes which control cellular proliferation and/or by the induced reactivation of genes, which are [normally] used only for embryological development, via demethylation. (Emphasis added)

23. Olek et al., in paragraph [0029], teach that genomic DNA can be isolated from stool samples. As stated therein:

> **[0029] The genomic DNA to be analyzed is obtained preferably from the usual sources for DNA, such as**, e.g., cell lines, blood, sputum, **stool**, urine, cerebrospinal fluid, tissue embedded in paraffin, for example, tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histological slides and all other possible combinations thereof. (Emphasis added)

24. The aspect of one testing DNA derived from a fecal sample so to determine if there is hypermethylation present is deemed to fairly suggest limitations of claims 96 and 97.

25. While Olek et al., teach performing tests on stool samples whereby one detects DNA sequence, they have not been found to teach testing for hemoglobin.

26. Kutzner et al., disclose method for the detection of colorectal tumors using a combination of tests- one which looks for a component of blood, and one which is based on nucleic acid analysis, e.g., PCR.

27. A seen therein, the faecal occult blood test was "a standard guaiac test", which is not an immunological test, deemed deficient, and does not seek to determine the amount of hemoglobin present.

28. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the methods and related devices of Wang et al., and Liang et al., so to include the testing of fecal samples for hemoglobin, hypermethylation/aberrant methylation and nucleic acid analysis, e.g., assaying for tumor genes. As evidenced above, Kutzner et al., teaches performing different types of assays on the fecal samples. By having different portions of a common fecal sample being produced and directed to the relevant lab, multiple assays can be performed in a more rapid pace, therein providing medical care in a more expeditious manner.

29. In view of the well-developed state of the art, said ordinary artisan would have had a most reasonable expectation of success.

30. In view of the above analysis and in the absence of convincing evidence to the contrary, claims 82, 84, 87, 96, and 97 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41).

31. Claims 89-94 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41), as applied to claims 82, 84, 87, 96, and 97, and further in view of US 2006/0188939 A1 (Gao) and Yeh (US 2008/0227298 A1).

32. See above for the basis of the rejection as it pertains to the disclosures of US 2006/0210448 A1 (Wang et al.), US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41).

33. While the prior art teaches detection of hemoglobin in a stool sample, they have not been found to teach that one can employ "immunochemical detection" (claim 89), nor disclose what concentration of hemoglobin detected is considered to be positive.

34. Gao teaches methods for detecting analytes in a fecal sample. As seen in paragraph [0041], such includes an immunoassay for fecal occult blood. Such a showing is deemed to fairly suggest limitation of claim 89.

35. While Gao teaches detecting hemoglobin in a fecal sample, Gao has not been found to teach the concentration of hemoglobin that can be detected.

36. Yee et al., paragraph [0041], teach setting a 10 ng/ml of hemoglobin in blood as a threshold value. Such a showing is deemed to meet a limitation of claims 90-94.

37. Applicant, at page 2 of the as-filed disclosure, under "BACKGROUND OF THE INVENTION," teaches that various methods, including immunochemical assays for detecting human hemoglobin in fecal sample was known in the art. As stated therein:

> Fecal occult blood tests fall primarily into two categories, tests based on the use of chromogenic chemical reagents such as gum guaiac and immunochemical tests. The chemically based guaiac methods determine the presence of occult blood by the detection of the perioxidase activity of the hemoglobin in the blood present in the faecal sample. They require catalysis of peroxide into oxygen and water, and the subsequent oxidation of a colorless dye (most often into a colored form). However, peroxidase activity is also found in meats and vegetables. In order to produce accurate results, these tests require restriction of the intake of certain foods, drugs, vitamins, and other substances prior to and during the sample collection period. The sensitivity of the most commonly used guaiac FOBT (Hemoccult) is approximately 50%. Despite a specificity of 98%, the positive predictive value for FOBT is low. Methods of detecting occult blood based on porphyrin (heme and protpoporphyrin IX) analysis or immunologic tests using anti-hemoglobin antibodies improve on these results**. Immunochemical tests (FIT or iFOBT) that use anti- hemoglobin antibodies specific for human blood in extracts from stool do not require dietary restrictions;** however, they are more complicated and more expensive than peroxidase-based tests. (Emphasis added).

38. It would have also been obvious to an ordinary artisan to have employed distinct portions from a subject's fecal sample in testing lab, as exemplified by Kutzner et al., to detect multiple signals indicative of colorectal cancer or tumor, e.g., an inclusion of detecting occult blood (hemoglobin) at various desired contractions based on known threshold levels. Said ordinary artisan would have been motived to have used immunochemicals/immunoassay for the detection of fecal hemoglobin as such methods and means of operation were clearly well known, thereby imparting a most reasonable expectation of success (Gao et al.). In light of the significant interest in testing patients' samples for such medically-significant target molecules, said ordinary artisan would have been amply motivated.

39. In view of the above analysis and in the absence of convincing evidence to the contrary, claims 89-94 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41), as applied to claims 82, 84, 87, 96, and 97, and further in view of US 2006/0188939 A1 (Gao) and Yeh (US 2008/0227298 A1).

40. Claims 98-100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41), as applied to claims 82, 84, 87, 96, and 97, and further in view of either Kim et al. (BMB reports, Volume 41, Issue 10, Pages 685-692, September 2008) or WO 2008/084219 A1 (Van England et al.).

41. See above for the bass of the rejection as it pertains to the disclosures of US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41)

42. Neither Wang et al., Liang et al., Olek et al., nor Kutzner et al., have been found to disclose the genes of claims 98 and 99, nor disclose the use of modifying DNA with bisulfite ions (claim 100).

43. Kim teaches colorectal cancer epigenetic biomarkers are used for the early detection of colorectal cancer with fecal-occult blood test (FOB). See page 686, Table 1, particularly column 1 bridging column 2 of page 689.

44. Applicant, page 5 of the disclosure, teaches that methods for detection of colorectal carcinoma based upon determining methylation of a number of different genes, including panels of genes, are disclosed in WO 2008/084219 A1 (Van England et al.).

> Van England relates to detection of colorectal cancer based upon determining methylation of a number of different genes, including panels of genes. The elected marker NDRG4 is also taught in Van England et al. (claims 1 and 2).

45. Applicant, at page 21, last paragraph, bridging to page 22 of the as-filed disclosure admits that numerous methods for assessing methylation are known in the art, including methods that comprise polymerase chain reaction (PCR), which is recognized as a form of amplifying DNA. Also admitted to therein is the use of chemical reagents so to modify the DNA prior to amplification was also well known in the art. As admitted to therein:

> **Determination of the methylation status may be achieved through any suitable means. Suitable examples include bisulphite genomic sequencing and/or by methylation specific PCR. Various techniques for assessing methylation status are known in the art and can be used in conjunction with the present invention**: sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR(McMS-PCR), MLPA with or without bisulphite treatment, QAMA (Zeschnigk et al, 2004), MSRE-PCR (Melnikov et al, 2005), MethyLight (Eads et al., 2000), ConLight-MSP (Rand et al., 2002), bisulphite conversion-specific methylation-specific PCR (BS-MSP)(Sasaki et al., 2003), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence diferences in PCR products of sodium bisulphite - treated DNA), methylation-sensitive single-nucleotide primer extension conformation(MS-SNuPE), methylation- sensitive single-strand conformation analysis (MS-SSCA), Melting curve combined bisulphite restriction analysis (McCOBRA)(Akey et al., 2002), PyroMethA, HeavyMethyl (Cottrell et al. 2004), MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles (QAMA), enzymatic regional methylation assay (ERMA), QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques for DNA methylation analysis is provided in Nucleic acids research, 1998, Vol. 26, No. 10, 2255-2264, Nature Reviews, 2003, Vol.3, 253-266; Oral Oncology, 2006, Vol. 42, 5- 13, which references are incorporated herein in their entirety. (Emphasis added)

46. Applicant, at page 24, third paragraph, admits that methods for performing MSP, wherein one employs primer pairs designed to distinguish methylated DNA from unmethylated DNA, was known in the art. As admitted to therein:

> In the MSP approach, DNA may be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulphite treatment (**Herman et al., 1996; and WO 97/46705**). For example, bisulphite ions modify non-methylated cytosine bases, changing them to uracil bases. Uracil bases hybridize to adenine bases under hybridization conditions. (Emphasis added)

47. The above showing is deemed to meet a limitation of claim 100.

48. Applicant, at page 26, last paragraph, bridging to page 27, of the as-filed disclosure further asserts:

> Whilst PCR is the preferred nucleic acid amplification technique, other amplification techniques may also be utilised to detect the methylation status of the concerned gene. Such amplification techniques are well known in the art, and include methods such as NASBA (Compton, 1991), 3SR (Fahy et al., 1991) and Transcription Mediated Amplification (TMA). Other suitable amplification methods include the ligase chain reaction (LCR) (Barringer et al, 1990), selective amplification of target polynucleotide sequences (US Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (US Patent No 4,437,975), arbitrarily primed polymerase chain reaction (WO 90/06995), invader technology, strand displacement technology, and nick displacement amplification (WO 2004/067726). This list is not intended to be exhaustive; any nucleic acid amplification technique may be used provided the appropriate nucleic acid product is specifically amplified. Thus, these amplification techniques may be tied in to MSP and/or bisulphite sequencing techniques for example. (Emphasis added)

49. In view of the above showing, it would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the methods of Liang et al., Olek et al., Kutzner et al. in view of Shuber, whereby one was detecting known, informative genes, such as the elected NDG4, TFPI2, PHACTR3, SFRP2 and SOX17, whereby one was using different portions of a faecal sample for the immunoassay and the nucleic acid-based assay, e.g., hypermethylation detection assays. It would have also been obvious to said ordinary artisan to

have employed means of stabilizing the sample portions for to do so would have a direct an immediate effect of being able to accurately and reproducibly determine the level of hemoglobin present, as well as preserve any nucleic acid present in the cells, and therein enhancing the ability to obtain accurate results.

50. In view of the teaching of the combined cited art such as Kutzner and Kim, which teaches a desire for accurate detection of early colorectal cancer with usages of various known molecular DNA biomarkers in addition with the FOBT, one of ordinary skilled artisans would have been motivated have employed admittedly known methods so to detect not only occult blood (hemoglobin) but known and useful gene targets, and to also determine whether the target sequences such as NDRG4 also demonstrate hypermethylation, be it in a gene of interest or a promoter of same.

51. In view of the well-developed state of the art and significant interest in being able to detect cancer at an early stage via both immunochemistry and nucleic acid analysis, said ordinary artisan would have been both well motivated and would have had a most reasonable expectation of success.

52. In view of the above analysis and in the absence of convincing evidence to the contrary, claims 98-100 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over US 2006/0210448 A1 (Wang et al.) in view of US 2009/0004058 A1 (Liang et al.), US 2005/0064401 A1 (Olek et al.) and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41), as applied to claims 82, 84, 87, 96, and 97, and further in view of

either Kim et al. (BMB reports, Volume 41, Issue 10, Pages 685-692, September 2008) or WO 2008/084219 A1 (Van England et al.).

Conclusion

53. Objections and/or rejections which appeared in the prior Office action and which have not been repeated hereinabove have been withdrawn.

54. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

55. A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

56. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Bradley L. Sisson whose telephone number is (571)272-0751. The examiner can normally be reached Monday to Thursday, from 6:30 AM to 5 PM..

57. Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is

encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

58. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on 571-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

59. Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Bradley L. Sisson/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost	
	Examiner Bradley L Sisson	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20060210448-A1	09-2006	Wang; Naishu	A61B10/0038	422/400
*	B	US-20090004058-A1	01-2009	Liang; Greg	B01L3/502	422/68.1
*	C	US-20080124714-A1	05-2008	Shuber; Anthony P.	C12Q1/6806	435/6.16
*	D	US-20060216830-A1	09-2006	Kikuiri; Hideki	B01L3/5082	436/165
*	E	US-20080227208-A1	09-2008	Yee; Hsiao-Ching	G01N33/72	436/66
*	F	US-20050064401-A1	03-2005	Olek, Alexander	C07K14/4703	435/6.12
*	G	US-20060188939-A1	08-2006	Gao; Ping	A61B10/0038	435/7.1
	H					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
*	N	WO-2008084219-A1	07-2008	WO	Van England et al.	A61P35/04
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Kutzner et al., "Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", Cancer Letters" 229 (2005) 33-41. (Year: 2005)
*	V	Kim et al., BMB reports, Volume 41, Issue 10, Pages 685-692, September 2008. (Year: 2008)
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	Art Unit
	Bradley L Sisson	1634

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021	03/22/2022			
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CLAIM		DATE								
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	85	÷	-	-	-	-	-			
	86	÷	-	-	-	-	-			
	87	÷	✓	✓	✓	✓	✓			
	88	÷	✓	✓	✓	✓	-			
	89	÷	✓	✓	✓	✓	✓			
	90	÷	✓	✓	✓	✓	✓			
	91	÷	✓	✓	✓	✓	✓			
	92	÷	✓	✓	✓	✓	✓			
	93	÷	✓	✓	✓	✓	✓			
	94	÷	✓	✓	✓	✓	✓			

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021	03/22/2022			
	95	÷	✓	✓	✓	✓	-			
	96	÷	✓	✓	✓	✓	✓			
	97	÷	✓	✓	✓	✓	✓			
	98	÷	✓	✓	✓	✓	✓			
	99	÷	✓	✓	✓	✓	✓			
	100	÷	✓	✓	✓	✓	✓			

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	Art Unit
	Bradley L Sisson	1634

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (see transcript for databases and search terms)	06/23/2019	B. L. S.
Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.
EAST (see transcript for databases and search terms)	01/14, 15/2020	B. L. S.
STN (see transcript for databases and search terms)	01/14/2020	B. L. S.
EAST (updated)	08/04/2020	B. L. S.
EAST	06/16/2021	B. L. S.
EAST	03/22/2022	B. L. S.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	Art Unit
	Bradley L Sisson	1634

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	128	DNA same "epigenetic modification" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/22 17:47
L2	1	DNA same "epigenetic modification" and (faecal fecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/22 17:47
L3	20	DNA same "epigenetic modification" and (faecal fecal stool) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/22 17:48
L4	21	DNA same "epigenetic modification" and (faecal fecal stool feces) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/22 17:48
L5	19	DNA same "epigenetic modification" and (dna "nucleic acid" chromosomal chromatin) same (faecal fecal stool feces) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/22 17:49
L6	0	"20050064401" and (hemoglobin haemoglobin)	US-PGPUB	OR	OFF	2022/03/22 18:07
L7	112	immunoassay same (hemoglobin haemoglobin) and @pd<"20090203"	US-PGPUB	OR	OFF	2022/03/22 18:33
L8	51	immunoassay with (hemoglobin haemoglobin) and @pd<"20090203"	US-PGPUB	OR	OFF	2022/03/22 18:33
L9	2	immunoassay with (hemoglobin haemoglobin) same (stool fecal faecal) and @pd<"20090203"	US-PGPUB	OR	OFF	2022/03/22 18:44
S1	53	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:50

S2	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 10:51
S3	9	(hemoglobin haemoglobin) with concentration same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:18
S4	260	DNA same ((stool fecal faecal) adj sample) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 11:35
S5	2	"20080227208"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:34
S6	1	"20080227208" and "10 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:35
S7	11	(hemoglobin haemoglobin) with "5 ng/ml"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2019/06/23 13:38
S8	0	NDGR4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S9	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:50
S10	6	NDRG4 same epigenetic and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 13:58
S11	24	"2006113770"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	OFF	2020/01/14 14:02

			DERWENT; IBM_TDB			
S12	4	"2006113770" and NDRG4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:11
S13	6	NDRG4 same (epigenetic methylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S14	6	NDRG4 same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:19
S15	0	"N-myc downstream regulated gene 4" same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S16	0	"N-myc downstream regulated gene 4" and (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:24
S17	0	"N-myc downstream regulated gene 4" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:25
S18	1	"20060019256" and uptg	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 14:55
S19	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/14 15:35
S20	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:07

S21	3	fecal same (antibody antibodies immunoassay) same (DNA RNA polynucleotide "nucleic acid" gene) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 08:26
S22	2	(cancer carcinoma) same (PHACTR3 NDRG4 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFRP2 CDO1) same (hyridization hybridizes hybridize) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:40
S23	1	"200483399"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:42
S24	0	"200483399A2"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/01/15 10:43
S25	0	"200483399A2"	EPO	OR	OFF	2020/01/15 11:11
S26	0	"200483399"	EPO	OR	OFF	2020/01/15 11:11
S27	40	(PHACTR3 FOXE1 GATA4 GPNMB TFP12 SOX17 SYNE1 LAMA MMP2 OSMR SFR2CDO1) same (epigenetic methylation hypermethylation) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/08/04 18:14
S28	38	NDRG4 and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2020/08/04 19:10
S29	15	hemoglobin with (stool fecal) with concentration and @pd<"20090203"	USPAT	OR	OFF	2021/06/15 10:13
S30	32	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) and (antibody antibodies immunoassay immunological) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 11:33
S31	16	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same (antibody antibodies immunoassay immunological) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 11:33

S32	1	(hemoglobin haemoglobin) with concentration same (stool fecal faecal) same (antibody antibodies immunoassay immunological) and "ng/ml" and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 11:37
S33	1,048	(freeze freezing frozen) same (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:05
S34	399	(freeze freezing frozen) with (stool fecal faecal) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:05
S35	236	(freeze freezing frozen) with (stool fecal faecal) with (sample fraction portion historical) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:07
S36	14	(freeze freezing frozen) with (stool fecal faecal) with (fraction portion historical) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:07
S37	4	(immunoassay elisa) with (stool fecal faecal) with (fresh "non-frozen") and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 12:31
S38	1	"7832219".FMID.	US-PGPUB; USPAT; FPRS; JPO	OR	OFF	2021/06/16 12:32
S39	4	"7832219"	USPAT	OR	ON	2021/06/16 12:33
S40	2	(freeze freezing frozen) with (stool fecal faecal) with (fraction portion historical) same buffer and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 13:30
S41	5	(freeze freezing frozen) same (stool fecal faecal) with (fraction portion historical) same buffer and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/06/16 13:33
S42	230	"5741650"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2021/06/16 13:38

			DERWENT; IBM_TDB			
S43	114	"5741650"	USPAT	OR	ON	2021/06/16 13:38
S44	63	"5741650" and frozen	USPAT	OR	ON	2021/06/16 13:40
S45	65	"5741650" and (freeze freezing frozen)	USPAT	OR	ON	2021/06/16 13:41
S46	3	(feces fecal faecal faeces) with buffer with (freeze freezing frozen) and @pd<"20090203"	USPAT	OR	ON	2021/06/16 13:51
S47	0	fecal with sample same (unfrozen "not frozen") and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/17 14:40
S49	85	fecal with sample same (frozen freezing) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/17 14:42
S50	12	fecal with sample same (sealable sealed seal) with (vessel container) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/17 14:57
S51	17	fecal with sample same (unfrozen "not frozen" freezing) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/21 15:56
S52	4	"20080124714"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/21 15:58
S53	6	fecal with sample same (sealable sealed seal) with (vessel container) same portion and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/21 16:18
S54	179	(stool fecal faecal) with sample with portion and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/21 16:52

S55	2	(stool fecal faecal) with sample with portion with (multiple duplicate) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/03/21 16:54
S56	69,546	((dna with (isolate extract extracted isolating)) (determine determining determins determined) with (hemoglobin haemoglobin) same (fecal faecal)) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/21 17:27
S57	1	((dna with (isolate extract extracted isolating)) and (determine determining determins determined) with (hemoglobin haemoglobin) same (fecal faecal)) and @pd<"20090203"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2022/03/21 17:28

3/22/2022 7:27:38 PM
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CONFIRMATION NO. 1874

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/634,607	06/27/2017 RULE	435	1634	EXCTD-35239.304

APPLICANTS

EXACT SCIENCES DEVELOPMENT COMPANY, LLC, Madison, WI;

INVENTORS

Joost Louwagie, Dornach, SWITZERLAND;

**** CONTINUING DATA ***************************

This application is a CON of 15/010,436 01/29/2016 ABN
which is a CON of 13/147,570 03/12/2012 ABN
which is a 371 of PCT/GB2010/000180 02/03/2010
which claims benefit of 61/149,581 02/03/2009

**** FOREIGN APPLICATIONS ***************************

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07/12/2017

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ Yes ☑ No	☐ Yes ☑ No	/BRADLEY L SISSON/ Examiner's Signature	☐ Initials	SWITZERLAND	0	19	2

ADDRESS

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562
UNITED STATES

TITLE

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
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Application	Document	Mailroom Date	Attorney Docket No.
15634607	CTFR	03/25/2022	EXCTD-35239.304
	892	03/25/2022	EXCTD-35239.304

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Doc Code: A.NE.AFCP

Document Description: After Final Consideration Pilot Program Request

PTO/SB/434 (05-13)

CERTIFICATION AND REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Practitioner Docket No.:	Application No.:	Filing Date:
EXCTD-35239.304	15/634,607	06/27/2017
First Named Inventor:	**Title:**	
Joost Louwagie	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD	

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0 (AFCP 2.0) OF THE ACCOMPANYING RESPONSE UNDER 37 CFR 1.116.

1. The above-identified application is (i) an original utility, plant, or design nonprovisional application filed under 35 U.S.C. 111(a) [a continuing application (*e.g.*, a continuation or divisional application) is filed under 35 U.S.C. 111(a) and is eligible under (i)], or (ii) an international application that has entered the national stage in compliance with 35 U.S.C. 371(c).
2. The above-identified application contains an outstanding final rejection.
3. Submitted herewith is a response under 37 CFR 1.116 to the outstanding final rejection. The response includes an amendment to at least one independent claim, and the amendment does not broaden the scope of the independent claim in any aspect.
4. This certification and request for consideration under AFCP 2.0 is the only AFCP 2.0 certification and request filed in response to the outstanding final rejection.
5. Applicant is willing and available to participate in any interview requested by the examiner concerning the present response.
6. This certification and request is being filed electronically using the Office's electronic filing system (EFS-Web).
7. Any fees that would be necessary consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, extension of time fees, are being concurrently filed herewith. [There is no additional fee required to request consideration under AFCP 2.0.]
8. By filing this certification and request, applicant acknowledges the following:
    - Reissue applications and reexamination proceedings are not eligible to participate in AFCP 2.0.
    - The examiner will verify that the AFCP 2.0 submission is compliant, *i.e.*, that the requirements of the program have been met (see items 1 to 7 above). For compliant submissions:
        - The examiner will review the response under 37 CFR 1.116 to determine if additional search and/or consideration (i) is necessitated by the amendment and (ii) could be completed within the time allotted under AFCP 2.0. If additional search and/or consideration is required but cannot be completed within the allotted time, the examiner will process the submission consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, by mailing an advisory action.
        - If the examiner determines that the amendment does not necessitate additional search and/or consideration, or if the examiner determines that additional search and/or consideration is required and could be completed within the allotted time, then the examiner will consider whether the amendment places the application in condition for allowance (after completing the additional search and/or consideration, if required). If the examiner determines that the amendment does not place the application in condition for allowance, then the examiner will contact the applicant and request an interview.
            - The interview will be conducted by the examiner, and if the examiner does not have negotiation authority, a primary examiner and/or supervisory patent examiner will also participate.
            - If the applicant declines the interview, or if the interview cannot be scheduled within ten (10) calendar days from the date that the examiner first contacts the applicant, then the examiner will proceed consistent with current practice concerning responses after final rejection under 37 CFR 1.116.

Signature	Date
/Mary Ann D. Brow/	2022-05-25
Name (Print/Typed) Mary Ann D. Brow	**Practitioner Registration No.** 42363

***Note**: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.*

☑ * Total of 1 forms are submitted.

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4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PATENT
Attorney Docket No.: **EXCTD-35239.304**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Joost Louwagie	Confirmation:	1874
Serial No.:	15/634,607	Group No.:	1634
Filed:	06/27/2017	Examiner:	Bradley L. Sisson
Entitled:	**FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**		

AMENDMENT UNDER 37 C.F.R. §1.116 AFTER FINAL OFFICE COMMUNICATION MAILED 03/25/2022 AND REQUEST FOR AFTER-FINAL CONSIDERATION UNDER AFCP 2.0

EFS WEB FILED
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This communication is an After Final Amendment under 37 C.F.R. §1.116(b)(2), responsive to the Final Office Communication mailed 03/25/2022, with a response due on or before 06/25/2022, and is filed with a Request for After-Final Consideration. Applicants respectfully request entry of this amendment.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: **EXCTD-35239.304**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Amendments to the Claims are reflected in the Listing of Claims beginning on Page 2;

Remarks begin on Page 5.

AMENDMENTS TO THE CLAIMS:

This listing of the claims will replace all prior listings and versions of claims in the application:

1-81. (canceled)

82. (currently amended) A method of processing a freshly-collected fecal sample without freezing, the method comprising:

a) collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject by defecation directly into a sealable collection vessel;

b) removing a portion of said fecal sample to a separate sealable container to produce a removed portion and a remaining portion of said fecal sample;

c) combining the removed portion in the separate sealable container with a buffer that prevents denaturation or degradation of blood proteins found in a fecal sample, and sealing the sealable container; and

d) stabilizing the remaining portion of the fecal sample by adding a buffer that maintains DNA integrity in a fecal sample to the remaining portion in the sealable collection vessel, and sealing the sealable collection vessel.

83. (canceled)

84. (currently amended) The method of claim 82, further comprising delivering the sealable container containing the removed portion and buffer and the sealable collection vessel containing the remaining portion of the fecal sample and buffer to a medical diagnostics laboratory.

85-86. (canceled)

87. (currently amended) A method of processing a fecal sample, the method comprising:

A) obtaining a pair of portions of a fecal sample from a human subject, the pair of portions comprising:

i) a sealed sealable container containing a stabilized removed portion and a buffer, and

ii) a sealed sealable collection vessel containing a stabilized remaining portion of a fecal sample and a buffer,

the pair of portions obtained by the method of claim 82,

B) testing the removed portion of said fecal sample for a concentration of hemoglobin present in said removed portion,

C) extracting DNA from the remaining portion of said fecal sample; and

D) analyzing said DNA for the presence of DNA from the human subject having an epigenetic modification.

88. (canceled)

89. (previously presented) The method of claim 87, wherein testing for the concentration of hemoglobin in said removed portion comprises immunochemical detection of hemoglobin.

90. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 5 ng/ml.

91. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 10 ng/ml.

92. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 20 ng/ml.

93. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 50 ng/ml.

94. (previously presented) The method of claim 87, wherein the removed portion of said fecal sample is considered positive for the presence of blood when the concentration of hemoglobin detected in said removed portion is at least 200 ng/ml.

95. (canceled)

96. (previously presented) The method of claim 87, wherein said epigenetic modification comprises aberrant methylation.

97. (previously presented) The method of claim 87, wherein said aberrant methylation comprises hypermethylation.

98. (previously presented) The method of claim 87, wherein said DNA from the human subject comprises a gene and/or a promoter region of a gene.

99. (previously presented) The method of claim 98, wherein said gene is selected from the group consisting of PHACTR3, NDRG4, FOXE1, GATA4, GPNMB, TFPI2, SOX17, SYNE1, LAMA, MMP2, OSMR, SFRP2, and CDO1.

100. (previously presented) The method of claim 96, wherein analyzing said DNA comprises modifying said DNA with bisulfite ions under conditions wherein unmethylated cytosine is converted to uracil.

REMARKS

In the Final Office Action mailed 03/25/2022 ("Office Action"), the Examiner made the following objections and rejections:

I. Claims 82, 84, 87, 96, and 97 are rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over US 2006/0210448 Al ("Wang") in view of US 2009/0004058 Al ("Liang"), US 2005/0064401 Al ("Olek") and Kutzner et al. ("Non-invasive detection of colorectal tumors by the combined application of molecular diagnostics and the faecal occult blood test", *Cancer Letters"* 229 (2005) 33-41; "Kutzner").

II. Claims 89-94 are rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wang in view of Liang, Olek, and Kutzner, as applied to claims 82, 84, 87, 96, and 97, and further in view of US 2006/0188939 Al ("Gao") and US 2008/0227208 Al ("Yee")[1].

III. Claims 98-100 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly allegedly being unpatentable over Wang in view of Liang, Olek, and Kutzner, as applied to claims 82, 84, 87, 96, and 97, and further in view of either Kim (BMB reports, Volume 41, Issue 10, Pages 685-692, September 2008; hereinafter "Kim") or WO 2008/084219 Al ("Van Engeland").

Claim amendments

For business reasons and without acquiescing to the Examiner's arguments, and reserving the right to prosecute the original or similar claims in one or more future applications, claim 82 is herein amended to recite removing a portion of the fecal sample to a separate sealable container, and further to recite, after combining the removed portion with the recited buffer, sealing the

[1] In paragraphs 31 and 39 of the Office Action, the Examiner cited "Yeh (US 2008/0227298 Al)." Applicant believes the Examiner intended to cite US 2008/0227208 A1 to Yee, as the cited publication number names inventor Hirota and is directed to semiconductor device manufacturing. If the Examiner indeed meant to cite US 2008/0227298 A1 to Hirota, Applicant requests correction of the Office Action.

sealable container. Claim 82 is further amended to recite in part d), after adding the recited buffer, sealing the sealable collection vessel. Sealing of the sealable container containing the buffer-stabilized removed portion and the collection vessel containing the buffer-stabilized remaining portion is demonstrated, *e.g.*, in Example 1, in the paragraph bridging pages 43 and 44, as evidenced by the shipping of the separate buffer-stabilized portions of the collected fecal samples, the shipping necessitating sealing of the containers containing the samples.

Claims 84 and 87 are amended for consistency with claim 82.

These amendments do not add new matter.

Applicant respectfully requests entry of these amendments in accordance with 37 C.F.R. 1.116 (b)(2).

The claims are not obvious

I. Claims 82, 84, 87, 96, and 97 are rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wang in view of Liang, Olek, and Kutzner. Applicant respectfully disagrees that the cited references render the claims *prima facie* obvious.

It is well-settled law that establishing *prima facie* obviousness requires *at least* a suggestion of all of the features in a claim. *See In re Wada and Murphy, citing CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) and *In re Royka*, 490 F.2d 981, 985 (CCPA 1974)). See also MPEP §2143. Further, a valid *prima facie* case requires the Office to present "articulated reasoning with some rational underpinning", *KSR*, 550 U.S. at 418 (citing *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)), that one of skill in the art would have been motivated to combine the elements to yield the claimed combination and that one would have done so having a reasonable expectation of successfully carrying out the claimed combination. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991).

As discussed above, the instant claims as amended are directed to a method of processing a freshly-collected fecal sample from a subject without freezing, wherein the sample is collected at home by the subject by defecation directly into a sealable collection vessel, wherein the sample is divided into portions that are separately stabilized, *e.g.*, for preserving different types

of analytes in each portion. In particular, a removed portion in a separate container is combined with a buffer that stabilizes blood proteins and the remaining portion is separately treated in the original collection vessel with a buffer that maintains DNA integrity in the fecal sample. A fecal sample collected in the claimed manner is suitable for detecting blood proteins in one portion and DNA in the other portion of the same fecal sample.

Applicant's claim 82 recites use of two different kinds of sealable containers:

1) a sealable collection vessel, and
2) a sealable container

As set forth in claim 82, the method requires that a fecal sample collected be at home by a human subject by defecation directly into the sealable collection vessel. The claim further recites that, after removal of the "removed portion" of the fecal sample, the "remaining portion" of the fecal sample is stabilized by addition of a buffer to the sealable collection vessel.

Claim 87 recites a pair of portions obtained by the method of claim 82, the pair of portions comprising:

i) a sealed sealable container containing a stabilized removed portion and a buffer, and
ii) a sealed sealable collection vessel containing a stabilized remaining portion of a fecal sample and a buffer.

The Examiner points to Wang as teaching a device for collecting a portion of a fecal sample and use of a preserving fluid, but admits that Wang does not teach collecting pairs of samples or sending pairs of samples to a lab for testing (Office Action, ¶17).

Applicant respectfully points out that Wang also fails to teach use of a sealable collection vessel as set forth in instant claims 82 and 87, collection of a sample by direct defecation into the sealable collection vessel, stabilization of a "remaining portion" of a fecal sample by buffer in the sealable collection vessel, or delivery of the sealable collection vessel containing the buffer-stabilized sample together with the buffer-stabilized removed portion to a lab for testing, as recited in claim 84.

Instead, the only sample collection device contemplated by Wang is suited only for

removing a small portion of a fecal sample using "stick 18", as illustrated in Figs. 1 and 2 of Wang, and having a "distal portion 21" having a groove to hold a small portion of a fecal sample (see, e.g., Wang at ¶25).

To the extent that Wang teaches catching a fecal sample using a toilet, Wang teaches that the catching is only for the purpose of temporarily catching the sample so that a portion can be removed by the disclosed stick device. For this purpose, Wang teaches providing a disposable paper catch web "temporarily secured to a toilet droopingly spanning the seat to catch the feces for sampling" (Office Action ¶15, referencing the Abstract of Wang). Wang specifies that the disposable web 61 is preferably made of a flushable paper (Wang, ¶34. See also Wang Fig. 7). Thus, the disposable web 61 of Wang is not a "sealable collection vessel" that can hold a buffer. Further, Wang does not suggest either preserving the remainder of the feces after collection of a portion using the disclosed sampling device, nor sending the disposable web 61 with the feces to a laboratory. Rather, Wang clearly points to disposing of the remaining feces, preferably by flushing it away with disposable web 61.

Further, the deficiencies of Wang with respect to using a combination of a sealable collection vessel and a sealable container as set forth in instant claims 82 and 87 is not remedied by combination of Wang with any of the other cited references.

The Examiner points to Liang for a teaching of a device and method for collecting and testing fecal samples (Office Action, ¶18). However, even if Liang discloses collection of multiple samples using several of the collection devices, as asserted by the Examiner, Liang suffers from the same deficiencies as Wang. Liang discloses only collecting small portions of feces using a "sampling wand" of the disclosed device. (Liang, ¶112).

Liang teaches that the user collects his/her feces on a "flushable paper sheet secured across the seat of a toilet," and uses a sampling wand inserted into the feces to collect the small portions for testing. The flushable paper sheet of Liang is not a "sealable collection vessel" that can hold a buffer. Further, Liang does not suggest either preserving the remainder of the feces after collection of a portion using the disclosed sampling wand, nor sending the flushable paper sheet with the feces to a laboratory. Rather, Liang clearly points to disposing of the remaining feces, preferably by flushing it away with flushable paper sheet.

Liang does not teach or suggest use of a sealable collection vessel as set forth in instant

claims 82 and 87, collection of a sample by direct defecation into the sealable collection vessel, stabilization of a "remaining portion" of a fecal sample by buffer in the sealable collection vessel, or delivery of the sealable collection vessel containing the buffer-stabilized sample together with the buffer-stabilized removed portion to a lab for testing as recited in claim 84. Thus, no matter how many of the devices of Wang and/or Liang are used to collect samples from feces, the combination of Wang and Liang still fails to teach of suggest all the elements of claims 82, 84 and 87 and the claims depending therefrom.

The Examiner points to Kutzner as disclosing detection of colorectal tumors using a combination of tests, one which looks for a component of blood, and one which is based on nucleic acid analysis, e.g., PCR analysis. (Office Action, ¶14).

While not acquiescing to the Examiner's characterization of Kutzner, Applicant points out that Kutzner describes a retrospective study of mutations and blood in frozen patient samples (Kutzner, p34, col 2). Kutzner does not teach or suggest a method of processing a freshly-collected fecal sample without freezing using a method comprising collection of a fecal sample at home by a human subject by defecation directly into a sealable collection vessel, or wherein a portion of the fecal sample is removed to a separate sealable container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample, as recited in instant claim 82, delivery of the containers to a laboratory as recited in claim 84, nor processing of such samples as set forth in claim 87.

Further, as acknowledged by the Examiner, Kutzner also does not suggest testing a fecal sample for a concentration of hemoglobin, (Office Action, ¶33), much less testing a removed portion collected as described in the instant claims for a concentration of hemoglobin. Kutzner also does not teach assaying DNA for aberrant methylation.

Kutzner clearly states that their "retrospective data *do not allow any conclusion* on the potential of this approach [combined application of molecular diagnosis (MD) and fecal occult blood test (FOBT)] in diagnostic screening" and state that "Prospective comparative studies, which include a very high number of individuals, have to prove the value of the combined application of MD and FOBT." (Kutzner, p40, paragraph bridging columns 1-2, emphasis added). Thus, Kutzner specifically states that it is not obvious to the authors of the reference, as

skilled artisans, that combined testing of MD markers with FOBT will yield any particular result with respect to diagnostic screening. Kutzner provides no more than a guess that the approach *might be* successful, but does not provide a "reasonable expectation of success" required to establish *prima facie* obviousness.

The Examiner points to Olek as teaching performing tests on stool samples whereby one detects DNA sequence. (Office Action, ¶22-25). However, Olek does not teach anything about either testing for hemoglobin, nor methods of collecting or processing fecal samples as set forth in Applicant's claims 82 and 87 and the claims depending therefrom. Olek therefore cannot cure the deficiencies of Wang in combination with Liang and Kutzner with respect to the missing elements.

While not acquiescing that the other requirements for establishing *prima facie* obviousness have been met, Applicant submits that, for the reasons set forth above, the combination of Wang in view of Liang, Olek, and Kutzner does not set forth the elements of Claims 82, 84, 87, 96, and 97 and, for at least this reason, does not render these claims *prima facie* obvious. Applicant therefore respectfully requests that the rejection be withdrawn.

II. Claims 89-94 are rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wang in view of Liang, Olek, and Kutzner, as applied to claims 82, 84, 87, 96, and 97, and further in view of Gao and Yee.

Applicant disagrees for at least the reason that Gao and Yee do not cure the deficiencies of Wang in view of Liang, Olek, and Kutzner, as applied to claims 82, 84, 87, 96, and 97.

The Examiner points to Gao as teaching methods of detecting analytes, including hemoglobin, in a fecal sample. The Examiner points to Yee as teaching setting a 10 ng/ml of hemoglobin in blood as a threshold value. (Office Action, ¶¶ 34-36).

However, neither Gao nor Yee teach or suggest a method comprising collection of a fecal sample at home by a human subject by defecation directly into a sealable collection vessel, or wherein a portion of the fecal sample is removed to a separate sealable container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample, as recited in

instant claims 82, or processing of such samples as set forth in claim 87. Claims 89-94 depend from claim 87 and thereby comprise the same elements.

Thus, while not acquiescing that the other requirements for establishing *prima facie* obviousness have been met, for the reasons set forth above Applicant submits that the combination of Wang in view of Liang, Olek, and Kutzner as applied to Claims 82, 84, 87, 96, and 97 and further in view of Gao and Yee fails to teach all the elements of claims 89-94 and thus does not render these claims *prima facie* obvious. Applicant therefore respectfully requests that the rejection be withdrawn.

III. Claims 98-100 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wang in view of Liang, Olek, and Kutzner, as applied to claims 82, 84, 87, 96, and 97, and further in view of either Kim or Van Engeland.

Applicant disagrees for at least the reason that Kim and Van Engeland do not cure the deficiencies of Wang in view of Liang, Olek, and Kutzner, as applied to claims 82, 84, 87, 96, and 97.

The Examiner errs in citing Van Engeland. (Office Action, ¶¶ 40, 44, 52). Applicant has previously established that Van Engeland, being filed less than one year prior to the priority date of the instant application could qualify as prior art *only* under pre-AIA 35 U.S.C. § 102(a). Applicant has further established by Affidavit that the Van Engeland does not disclose invention "by another," as required under pre-AIA 35 U.S.C. § 102(a), and therefore does not qualify as prior art to the present application. Applicant directs the Examiner to Applicant's Response filed 11/29/2021 and to the Inventor's Declaration Under 37 C.P.R. § 1.132 by inventor of the present invention Joost Louwagie ("Louwagie Decl."), which was originally filed with the Office in Appl. Ser. No., 13/147,570, to which the instant application claims priority as a continuation of U.S. Application No. 15/010,436, filed January 29, 2016, which is a continuation of U.S. Application No. 13/147,570.

Furthermore, even if Van Engeland is considered in the present rejection as disclosing the marker NDRG4, Van Engeland fails to cure the deficiencies of Wang in view of Liang, Olek, and Kutzner, discussed in detail above, with respect to the missing elements of the instant claims.

The Examiner points to Kim as allegedly teaching colorectal cancer epigenetic markers. (Office Action, ¶43). However, Kim does not cure the deficiencies of Wang in view of Liang, Olek, and Kutzner.

Examiner points to Kim as allegedly teaching "colorectal cancer epigenetic biomarkers are used for the early detection of colorectal cancer with fecal-occult blood test (FOB)" pointing to page 686, Table 1, particularly column 1 bridging column 2 of page 689. (Office Action, ¶43). Applicant respectfully disagrees with the Examiner's characterization of Kim, as Kim makes no mention of testing epigenetic biomarkers along with FOB *on the same fecal specimen*. Rather, Kim is a review article that catalogues the "noninvasive molecular biomarkers that are currently in clinical settings and have the potential for use as CRC biomarkers" as of the time Kim was published in 2008 (Kim, Abstract). While Kim references the NDRG4 marker, nothing in Kim teaches or suggests *combining* detection of NDRG4 (or any other colorectal cancer epigenetic biomarkers) with fecal-occult blood testing on separately stabilized portions of the same freshly collected fecal sample, as recited in Applicant's claims.

Further, Kim does not teach or suggest a method comprising collection of a fecal sample at home by a human subject by defecation directly into a sealable collection vessel, or wherein a portion of the fecal sample is removed to a separate sealable container and combined with a buffer that stabilizes blood proteins, while the portion remaining in the sealable collection vessel is combined with a buffer that maintains DNA integrity in the fecal sample, as recited in instant claims 82, or processing of such samples as set forth in claim 87. Claims 98-100 depend from claim 87 and thereby comprise the same elements.

Thus, while not acquiescing that the other requirements for establishing *prima facie* obviousness have been met, for the reasons set forth above Applicant submits that the combination of Wang in view of Liang, Olek, and Kutzner as applied to Claims 82, 84, 87, 96, and 97 and further in view of Kim fails to teach all the elements of claims 98-100 and thus does not render these claims *prima facie* obvious. Applicant therefore respectfully requests that the rejection be withdrawn.

Applicant's alleged admissions

In addition to the references discussed above, the Examiner characterized a number of

items from Applicant's Specification as applicant admissions. See, e.g., Office Action at ¶¶ 45-48. 46. While the alleged applicant admissions are not enumerated or discussed here, the absence of discussion in this response is not to be taken as a concession that any information from Applicant's Specification is conceded by the Applicant to be prior art to the instant claims, nor that the Examiner's characterization of any such alleged admission is correct.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that all grounds for rejection have been addressed and Applicant's claims should be passed to allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicant encourages the Examiner to call the undersigned at (608) 662-1277.

Dated: May 25, 2022 /Mary Ann D. Brow/

Mary Ann D. Brow
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Electronic Acknowledgement Receipt

EFS ID:	45802214
Application Number:	15634607
International Application Number:	
Confirmation Number:	1874
Title of Invention:	FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD
First Named Inventor/Applicant Name:	Joost Louwagie
Customer Number:	173254
Filer:	Mary Ann Deese Brow
Filer Authorized By:	
Attorney Docket Number:	EXCTD-35239.304
Receipt Date:	25-MAY-2022
Filing Date:	27-JUN-2017
Time Stamp:	14:49:42
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	After Final Consideration Program Request	2022-05-22_EXCTD-35239-304_AFCP_Form.pdf	226487 bb076be1285e9653f060373a541587726ca79346	no	2

Warnings:

Information:					
2		2022-05-25_RFOA_EXCTD-003 USCON2-35239_35239-304.pdf	174010 84f0ba537ce004cc19bb6085b87c4befc4b691b2	yes	13

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Response After Final Action	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	13

Warnings:	
Information:	
Total Files Size (in bytes):	400497

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/634,607	Filing Date 06/27/2017	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT	05/25/2022	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0		x $0 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $0 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/YOLANDA CHADWICK/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/634,607	06/27/2017	Joost Louwagie	EXCTD-35239.304	1874

173254 7590 06/14/2022

CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

EXAMINER	
SISSON, BRADLEY L	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
06/14/2022	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

Advisory Action
Before the Filing of an Appeal Brief

Application No.	Applicant(s)		
15/634,607	Louwagie, Joost		
Examiner	**Art Unit**	**AIA (FITF) Status**	
Bradley L Sisson	1634	No	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 25 May 2022 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☑ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☑ The period for reply expires 3 months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection.The current period for reply expires ____ months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.

   Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANTS FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37CFR 41.37(a).

AMENDMENTS

3. ☑ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☑ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: See Continuation Sheet (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☑ For purposes of appeal, the proposed amendment(s):(a) ☑ will not be entered, or (b) ☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☑ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☑ Other: See Continuation Sheet.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
    Claim(s) allowed:_____.
    Claim(s) objected to:_____.
    Claim(s) rejected:82,84,87,89-94 and 96-100.
    Claim(s) withdrawn from consideration:_____.

/Bradley L. Sisson/
Primary Examiner, Art Unit 1634

Continuation of 3. NOTE: The aspect of adding new limitation to independent claim 82 whereby the container is sealable would require further consideration and/or search

Continuation of REQUEST FOR RECONSIDERATION/OTHER 12. The request for reconsideration has been considered but does NOT place the application in condition for allowance because: Applicant's traversal of the rejection has been found to be predicated on the entry of the amendment. As indicated above, the amendment has not been entered. Therefore, the rejections are maintained for reasons of record.

Continuation of REQUEST FOR RECONSIDERATION/OTHER 14. Other: Attached:
a) PTO-892 Notice of references Cited
b) PTO-413 Interview Summary
c) PTO-2323 After-Final Consideration Pilot

Examiner-Initiated Interview Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost		
	Examiner Bradley L Sisson	Art Unit 1634	AIA (First Inventor to File) Status No	Page 1 of 2

All Participants (applicant, applicants representative, PTO personnel)	Title	Type
Bradley L Sisson	Primary Examiner	Telephonic
Mary Ann D. Brow, Reg. No. 42,363	Attorney of Record	

Date of Interview: 09 June 2022

Issues Discussed:

Proposed Amendment(s)

The amendment of 25 May 2022, filed under 37 CFR 1.116, was discussed. Ms. Brow directed attention to the limitation that a human defecates "directly into a sealable collection vessel", and that a "portion" of the fecal sample is removed to a "separate sealable container".

Examiner Sisson indicated that in conducting an updated search of prior art that several relevant documents had been identified. Those documents are:

a) US 2004/0019298 A1 (Zhou et al.), in the abstract and at paragraph [0025], teach collection of a fecal sample and that the collection device is loadable into a sealable shipping capsule.

b) US 6,761,702 B2 (Smith), in th abstract and at column 2, fifth paragraph, teaches of colonic lavage and that the fluid and cleansed fecal material is passed to a sealable container.

c) US 2005/0075543 A1 (Calabrese), at paragraph [0040], teaches of a kit for collection of sample at home, the use of a sealable container, and that "The urine or stool sample is collected y collecting enough of he specimen in the sample container." And that in paragraph [0052], it is disclosed that " Alternatively, the patient may use a home kit to obtain and submit the sample..."

d) US 2003/0086869 A1 (Stallings et al.), in paragraph [0086], teach of 72-hour stool collection and that it is collected and initially stored at home.

e) US 4,445,235 (Slover et al.) teaches of a collection device that inserts into a toilet.

f) US 2007/0017015 A1 (Finell), in paragraph [0082], teaches of a collection chamber in a potty, and that one defecates directly into the the chamber.

Examiner Sisson noted that the instant application had not been filed with any drawings/figures, much less figures that describe the "sealable collection vessel" that one is to defecate "directly into".

Ms. Brow directed attention to page 23, first paragraph, line 7. of the as-filed disclosure which refers to " a special bucket".

Examiner Sisson noted that while the specification may refer to "a special bucket", such does not describe how it is designed not only for direct defecation, but for sealing upon defecation and resealing of the "sealable collection vessel" (proposed claim 82) once a sample had been removed therefrom and placed into a separate sealable container.

Examiner-Initiated Interview Summary	Application No. 15/634,607	Applicant(s) Louwagie, Joost		
	Examiner Bradley L Sisson	Art Unit 1634	AIA (First Inventor to File) Status No	Page 2 of 2

While not discussed during he interview, attention is also directed to US 2008/0097238 A1 (Loktionov et al.), at paragraph [0013], teaches of isolating exfoliated cells from the surface of human stool. As stated therein:

"A method of exfoliated cell isolation from human whole stool samples by washing cells off the surface of cooled faeces and collecting them by immunomagnetic separation procedure has been developed [Loktionov, A. et al., Clin. Cancer Res. 1998; 4: 337-342]. Although work in this direction was initially planned in terms of developing a molecular diagnostic assay for CRC, it emerged that a simple quantitative analysis of colonocyte-derived DNA from human stool surface could be used for CRC diagnosis and screening since the relative DNA amount in CRC patients was much higher compared to healthy individuals."

Attention is also directed to US 2003/0096244 A1 (Rabello et al.), which teaches at paragraph [0037], of isolating from human feces cells from a parasite, and ultimately the DNA from within the parasitic cells, wherein the parasite is Schistosoma.

Such teachings arguably provide motivation for one to not only obtain a small, surface sample of fecal material for human DNA for signs of disease (e.g., colon cancer), as well as obtain and test the large sample for cells, and the DNA contained therein, for the detection of parasites in the larger sample portion.

Other

Ms. Brow indicated that applicant may file an appeal or file a pre-appeal brief review request and conference.

/Bradley L. Sisson/ Primary Examiner, Art Unit 1634	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Notice of References Cited	Application/Control No. 15/634,607	Applicant(s)/Patent Under Reexamination Louwagie, Joost	
	Examiner Bradley L Sisson	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20040019298-A1	01-2004	Zhou, David F.	A61B10/0096	600/564
*	B	US-6761702-B2	07-2004	Smith; Alan	A61M1/774	604/35
*	C	US-20050075543-A1	04-2005	Calabrese, Charles A.	G16H10/40	600/300
*	D	US-20030086869-A1	05-2003	Stallings, Virginia A.	C12Q1/44	424/9.1
*	E	US-4445235-A	05-1984	Slover; Pearl	A61B10/0038	4/144.1
*	F	US-20070017015-A1	01-2007	Finell; Rebecca M.	A47K11/06	4/483
*	G	US-20080097238-A1	04-2008	Loktionov; Alexandre	A61B10/0096	600/562
*	H	US-20030096244-A1	05-2003	Rabello, Ana Lucia Teles	C12Q1/6888	435/7.22
	I					
	J					
	K					
	L					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

AFCP 2.0 Decision	Application No.	Applicant(s)	
	15/634,607	Louwagie, Joost	
	Examiner	Art Unit	AIA (FITF) Status
	Bradley L Sisson	1634	No

This is in response to the After Final Consideration Pilot request filed 25 May 2022.

1. **Improper Request** – The AFCP 2.0 request is improper for the following reason(s) and the after final amendment submitted with the request will be treated under pre-pilot procedure.

   ☐ An AFCP 2.0 request form PTO/SB/434 (or equivalent document) was not submitted.

   ☐ A non-broadening amendment to at least one independent claim was not submitted.

   ☐ The request is not the first proper AFCP 2.0 request submitted in response to the most recent final rejection.

   ☐ Other: _____

2. **Proper Request**

   A. After final amendment submitted with the request will not be treated under AFCP 2.0.
   The after final amendment cannot be reviewed and a search conducted within the guidelines of the pilot program.

   ☐ The after final amendment will be treated under pre-pilot procedure.

   B. Updated search and/or completed additional consideration.
   The examiner performed an updated search and/or completed additional consideration of the after final amendment within the time authorized for the pilot program. The result(s) of the updated search and/or completed additional consideration are:

   ☐ 1. All of the rejections in the most recent final Office action are overcome and a Notice of Allowance is issued herewith.

   ☐ 2. The after final amendment would not overcome all of the rejections in the most recent final Office action . See attached interview summary for further details.

   ☑ 3. The after final amendment was reviewed, and it raises a new issue(s). See attached interview summary for further details.

   ☐ 4. The after final amendment raises new issues, but would overcome all of the rejections in the most recent final Office action. A decision on determining allowability could not be made within the guidelines of the pilot. See attached interview summary for further details, including any newly discovered prior art.

   ☐ 5. Other: _____

   Examiner Note: Please attach an interview summary when necessary as described above.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	Art Unit
	Bradley L Sisson	1634

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (see transcript for databases and search terms)	06/23/2019	B. L. S.
Reviewed 15/010436 & 13/147570	06/23/2019	B. L. S.
EAST (see transcript for databases and search terms)	01/14, 15/2020	B. L. S.
STN (see transcript for databases and search terms)	01/14/2020	B. L. S.
EAST (updated)	08/04/2020	B. L. S.
EAST	06/16/2021	B. L. S.
EAST	03/22/2022	B. L. S.
PE2E Search	06/09/2022	B. L. S.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
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✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021	03/22/2022	06/09/2022		
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
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CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021	03/22/2022	06/09/2022		
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	83	÷	✓	✓	✓	✓	-	-		
	84	÷	✓	✓	✓	✓	✓	✓		
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	86	÷	-	-	-	-	-	-		
	87	÷	✓	✓	✓	✓	✓	✓		
	88	÷	✓	✓	✓	✓	-	-		
	89	÷	✓	✓	✓	✓	✓	✓		
	90	÷	✓	✓	✓	✓	✓	✓		
	91	÷	✓	✓	✓	✓	✓	✓		
	92	÷	✓	✓	✓	✓	✓	✓		
	93	÷	✓	✓	✓	✓	✓	✓		
	94	÷	✓	✓	✓	✓	✓	✓		

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/634,607	Louwagie, Joost
	Examiner	**Art Unit**
	Bradley L Sisson	1634

CLAIM		DATE								
Final	Original	03/18/2019	06/23/2019	01/15/2020	08/04/2020	06/16/2021	03/22/2022	06/09/2022		
	95	÷	✓	✓	✓	✓	-	✓		
	96	÷	✓	✓	✓	✓	✓	✓		
	97	÷	✓	✓	✓	✓	✓	✓		
	98	÷	✓	✓	✓	✓	✓	✓		
	99	÷	✓	✓	✓	✓	✓	✓		
	100	÷	✓	✓	✓	✓	✓	✓		