Most techniques for improved sensitivity of cancer detection from faecal samples focus on improvements in recovery of target human DNA from the total DNA. The inventors have successfully improved the sensitivity of detection of colorectal cancer in faecal samples by increasing the amount of DNA used in the detection reactions. Increasing the amount of DNA in the detection reaction goes along with an increase in substances co-purified with the DNA. An increase in the amount of impurities may be expected to result in PCR-inhibition, and therefore an increased level of input DNA in the detection reaction has not been previously explored for improving the sensitivity of cancer detection in faecal samples.

Accordingly, in a further aspect, the invention provides a method of processing a faecal sample to isolate and prepare DNA for use in detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising:

(a) isolating DNA from the faecal sample

(b) subjecting at least 2.5µg of the isolated DNA per amplification reaction required to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues

(c) amplifying the treated isolated DNA.

Thus, the inventors have found that by including at least 2.5µg of isolated DNA in the reagent treatment step for every downstream amplification that is required, improved

detection methods using stool samples are achieved. The amount of DNA is expressed per amplification reaction required in particular to allow for multiple parallel reactions to be carried out on the same sample. For example, test and control samples can then be run in parallel. Also, where detection of an epigenetic change, preferably methylation, in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) is not carried out in the same reaction (by use of appropriate fluorophores for example) each of a panel of genes may be assessed in a separate reaction. Thus a single starting sample may need to be split into a plurality of sub-samples, as required. This improves the accuracy of the results obtained by minimizing inter-sample variations. The amount of isolated DNA per amplification reaction is at least approximately 2.5µg, 3µg, 4µg, 5µg, 7.5µg, 10µg etc. to improve sensitivity, and is most preferably approximately 2.5µg.

In one embodiment, the method further comprises, preferably prior to isolation of DNA from the sample, adding a homogenization buffer to the faecal sample. Any suitable buffer may be utilized. Useful buffers are commercially available, for example from Amresco.

The reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues the reagent preferably

comprises, consists essentially of or consists of a bisulphite reagent. Suitable reagents are discussed herein, which discussion applies *mutatis mutandis*. In specific embodiments, the bisulphite reagent comprises, consists essentially of or consists of sodium bisulphite.

In a specific embodiment, between treatment of the isolated DNA with the reagent and amplification of the treated isolated DNA, the treated isolated DNA is concentrated. Any suitable DNA concentration method may be utilised. For example, a DNA-binding reagent may be utilised in order to concentrate DNA from the sample. DNA-binding reagents may be selected from DNA-binding buffers, DNA-binding filters, DNA-binding columns etc. and may require use of a centrifugation step. Suitable kits are commercially available, such as the ZYMO Clean and Concentrator Kit available from Zymo Research.

In order to achieve the necessary recovery of DNA from the faecal sample, the faecal sample may be at least approximately 4g in weight. The faecal sample may be anywhere between approximately 2g and 10g in weight and is most preferably around 4g in weight.

The methods of the invention may thus include steps such as:

- obtaining and processing a stool sample from the subject under test, wherein preferably around (at least) 4g stool is obtained
- adding homogenization buffer, preferably directly after defecation (the subject may add this themselves). The buffer may be added at any suitable ratio, such as at 1:7 for example

- isolating DNA from the stool sample. As mentioned above, any suitable DNA isolation technique may be employed. This may involve (a double) low speed centrifugation. Isolation may require RNase A and Proteinase K treatment followed by DNA extraction, for example by phenol/chloroform extraction. Other DNA purification techniques can be used, as discussed herein
- subjecting the obtained DNA to bisulphite conversion, such as subjecting at least 18 to 32 µg DNA to bisulphite conversion or subjecting at least 2.5 µg DNA to bisulphite conversion for each PCR reaction to be done (input expressed per PCR reaction for reason of multiplexing as discussed earlier)
- concentrating the amount of bisulphite treated DNA obtained from the at least 18 to 32 µg untreated DNA
- amplifying the amount of bisulphite converted DNA. The amount to be amplified may be equivalent to 10 to 2.5 µg unconverted DNA (this equals the amounts for 4 marker panels down to use of a single marker).

The sensitivity of the methods for processing a faecal sample may be improved further by combining them with known methods for isolating DNA from a faecal sample. For example, the human DNA component may be purified from a stool sample using streptavidin-bound magnetic beads (Dong et al., 2001; Ahlquist et al., 2000). In a further embodiment, an electrophoresis-driven separation of target DNA sequences, using oligonucleotide capture probes immobilized in an acrylamide gel (Whitney et al., 2004) may be utilised in order to purify human DNA from the stool sample. In a still further embodiment, which may be used in the alternative or in combination with earlier embodiments,

faecal samples may be frozen as quickly as possible after collection in order to preserve DNA integrity. As discussed above, DNA integrity may also be usefully tested in terms of diagnosing colorectal cancer. Additionally or alternatively, stabilization buffer may be added to the faecal samples before transport of the samples (Olson et al., 2005). In a yet further complementary embodiment, Methyl-binding domain (MBD) protein may be utilised to enrich methylated human DNA from a faecal sample, in order to specifically improve sensitivity for detecting methylated DNA markers in the sample (Zou et al., Clin Chem. 2007 Sep;53(9):1646-51).

In specifc aspects, the methods of processing a faecal sample according to the invention are combined with the other methods of the invention in order to provide improved diagnosis, histopathological analysis, pharmaogenomic analysis etc. of colorectal cancer. Accordingly, all embodiments of the methods of the invention apply *mutatis mutandis* Thus, the methods of the invention can be performed on the amplified treated DNA to provide particularly sensitive methods relating to colorectal cancer for example.

In a still further aspect, the invention provides a method of determining the methylation status of at least one gene in a blood sample, in particular a blood plasma or serum sample, comprising:

(a) isolating DNA from a blood plasma or serum sample

(b) subjecting the isolated DNA to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues

(c) amplifying the treated isolated DNA in order to determine the methylation status of at least one gene, characterised in that 0.07 to 0.72 ml blood plasma or serum sample equivalent of DNA is used per amplification reaction.

The methods thus utilise small volumes in the amplification reactions yet still maintain high sensitivity and specificity of detection. Thus, as discussed herein, a single blood sample may be advantageously utilised to determine the methylation status of a panel of genes in one embodiment. The volumes may be anywhere between around 0.07 and around 0.72 ml blood plasma or serum equivalent, and as discussed below preferably plasma equivalent, of DNA per amplification reaction. In specific embodiments, between around 0.07, 0.10, 0.15 and 0.50, 0.60, 0.70 ml, such as between 0.07 and 0.15, 0.16, 0.17, 0.18 or 0.19 ml blood plasma or serum equivalent of DNA is used per amplification reaction. In a specific embodiment, substantially the same selected volumes of blood plasma or serum sample, equivalent of DNA is used for each amplification reaction carried out. Thus, where multiple amplifications are carried out based upon a single blood sample taken from a subject, each amplification will utilise 0.07 to 0.72 ml blood plasma or serum sample, equivalent of DNA.

The blood plasma or serum sample may be derived from whole blood or any suitable plasma or serum containing

parts/fractions thereof as appropriate. In specific embodiments, the blood plasma or serum sample comprises, consist essentially of or consists of plasma. The blood sample, from which the plasma or serum is derived may be collected using any suitable method Many such methods are well known in the art. In one embodiment, the methods of the invention also incorporate the step of obtaining the blood sample and/or the plasma or serum sample from whole blood. Any appropriate blood sample may be utilised in the methods of the invention, provided it contains sufficient (free floating) DNA. In a specific embodiment, the volume of the blood sample, or derivative thereof that is utilised in the methods is around 5 to 15 ml, such as 10 ml.

Blood samples, or derivatives thereof and in particular plasma or serum samples, may be stored prior to use in the methods of the invention once obtained. They may be frozen, for example, at a suitable temperature. Suitable temperatures may be between around 0°C, -1°C, -2°C, -3°C, -4°C and -20°C, -30°C, -40°C, -50°C, -60°C, -70°C, -80°C, -90°C etc., such as around -80°C. They may also be stored at other temperatures, such as at 4°C or at room temperature depending upon their form. In one specific embodiment, plasma or serum is dried to allow storage at non-freezing temperatures. The drying may comprise lyophilization for example, although other dehydration techniques may be employed. Where plasma or serum is stored at temperatures greater (i.e. warmer) than freezing, and in particular greater than -80°C, antimicrobial agents such as antibiotics may be added to the sample to prevent spoiling.

In one embodiment, stabilizers are added to the blood sample, or derivative thereof, in particular serum or plasma. This is particularly relevant where the sample is not frozen. In one specific embodiment, where the sample is serum, stabilizers such as stabilizers selected from EDTA and/or citrate and/or heparin are employed. In a further embodiment, where the sample is plasma, stabilizers such as stabilizers selected from citrate and/or heparin may be utilised.

It is preferred that the blood plasma or serum sample comprises, consists essentially of or consists of a plasma sample. Plasma may be derived from whole blood by any suitable means. In one embodiment, the plasma sample is obtained by centrifugation of whole blood. Centrifugation may be carried out at any suitable speed and for any suitable period of time and under any suitable conditions as may be determined by one skilled in the art. For example, centrifugation may be carried out at between around 1000 and 3000g. Centrifugation may be carried out for between around 1, 2, 3, 4, or 5 and 10, 11, 12, 13, 14 or 15 minutes for example. Centrifugation may be carried out at low temperatures, such as between around 0 and 5°C, for example 4°C, to maintain integrity of the sample. Multiple centrifugation steps may be employed in order to obtain the plasma sample. In a specific embodiment, two centrifugation steps are employed to obtain the plasma sample.

It has been shown that sensitivity of the methods of the invention may be improved by excluding samples with a plasma (or serum) volume less than around 1 to 3 ml and in particular around 2 ml (such as 1.5 to 2.5 ml) prior to

isolating DNA. Thus, the methods may comprise determining the volume of plasma (or serum) obtained from a blood sample prior to DNA isolation. If the volume of the plasma (or serum) obtained from the blood sample is less than around 1 to 3 ml and in particular around 2 ml (such as 1.5 to 2.5 ml), the sample is excluded from further assessment.

As stated herein, the methods are useful for determining the methylation status of at least one gene. By "determining the methylation status" is meant determining the presence or absence of 5-methylcytosine ("5-mCyt") at one or a plurality of (functionally relevant) CpG dinucleotides within the DNA sequence of the at least one gene. In particular, aberrant methylation, which may be referred to as hypermethylation, of the at least one gene may be detected. Typically, the methylation status is determined in one or more CpG islands in the at least one gene. These CpG islands are often found in the promoter region of the gene(s). Thus, CpG dinucleotides are typically concentrated in the promoter regions and exons of human genes and the methylation status of these CpG residues is of functional importance to whether the at least one gene is expressed. Since CpG dinucleotides susceptible to methylation are typically concentrated in the promoter region, exons and introns of human genes, promoter, exon and intron regionsmay be assessed in order to determine the methylation status of the at least one gene. A "promoter" is a region extending typically between approximately 1 Kb, 500 bp or 150 to 300 bp upstream from the transcription start site. The CpG island may surround or be positioned around the transcription start site of the at least one gene.

The methods of the invention involve isolating/extracting/purifying DNA from the blood plasma or serum sample. Any suitable DNA isolation technique may be utilised, as discussed herein, which discussion applies here mutatis mutandis. Likewise, suitable methods and kits for isolating DNA from blood samples which are commercially available are discussed and exemplified herein, which discussion applies here mutatis mutandis (see table 1). Thus, as can be derived from the table , DNA isolation may be carried out using silica-membranes, isopropanol or magnetic bead based methods for example.

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

The methods of this aspect of the invention (and other aspects of the invention which involve certain types of methylation detection) rely upon a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues. Any suitable reagent may be utilised in the methods of the

invention. Examples include bisulphite, hydrogen sulphite and disulphite reagents and suitable mixtures thereof. In an embodiment of the invention, the reagent comprises, consists essentially of or consists of a bisulphite reagent. In particular, the reagent may comprise, consist essentially of or consist of sodium bisulphite.

In a specific embodiment, following treatment of the isolated DNA with the reagent, and preferably between treatment of the isolated DNA with the reagent and amplification of the treated isolated DNA, the treated isolated DNA is concentrated. Any suitable DNA concentration method may be utilised. For example, a DNA-binding reagent may be utilised in order to concentrate DNA from the sample. DNA-binding reagents may be selected from DNA-binding buffers, DNA-binding filters, DNA-binding columns etc. and may require use of a centrifugation step. Suitable kits are commercially available, such as the ZYMO Clean and Concentrator Kit available from Zymo Research.

In one specific embodiment, the at least one gene whose methylation status is determined is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. As is discussed in detail herein, the methylation status of these genes in blood plasma or serum samples is correlated with the incidence of cancer and in particular colorectal cancer. Details of these genes are provided herein which discussion applies to this aspect mutatis mutandis.

In a specific embodiment, the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23 since these four genes have been shown to be particularly reliably linked to the

incidence of colorectal cancer using blood derived samples, in particular plasma sample.

Also, these genes have been shown to be linked to early stage colorectal cancer. Accordingly, the invention provides a method of determining the methylation status of at least one gene selected from OSMR, NDRG4, GATA5 and ADAM23 in a blood sample, in particular a blood plasma or serum sample, comprising:

(a) isolating DNA from a blood plasma or serum sample

(b) subjecting the isolated DNA to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues

(c) amplifying the treated isolated DNA in order to determine the methylation status of at least one gene, characterised in that 0.07 to 0.72 ml blood plasma or serum sample equivalent of DNA is used per amplification reaction.

This method may be utilised in order to diagnose early stage colorectal cancer, in particular stage 0 to II colorectal cancer. It may also be used to stage colorectal cancer - detection of methylated gene or genes indicates an early stage of cancer. Corresponding methods and kits are also envisaged. These methods may additionally or alternatively be usefully applied applied to determine the methylation status of at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, such as from TFPI2, BNIP3, FOXE1, STNE1 and SOX17, in particular TFPI2.

Moreover, in order to improve the sensitivity of the methods of the invention the methods may comprise determining the

methylation status of a panel of genes comprising at least two, three, four, five or six (of the) genes. Thus, in one embodiment, the at least one gene forms part of a panel of genes comprising at least two, three, four, five or six genes, wherein the methylation status of each of the genes is determined. The panel of genes may comprise, consist essentially of or consist of two, three, four, five or six genes. Suitable panels are discussed herein in respect of other aspects of the invention. That discussion and those embodiments apply here mutatis mutandis.

In specific embodiments, the panel of genes comprises, consists essentially of or consists of OSMR, NDRG4, GATA5 and ADAM23. This panel may be useful in the diagnosis of early stage colorectal cancer, such as stage 0 to II colorectal cancer.

It is noted that for each gene, it may be possible to determine the methylation status of the gene, in a plurality of locations within the same gene (as discussed herein). Thus, for example, a gene may incorporate more than one CpG island, or multiple sites within the same CpG island may be investigated as appropriate.

As discussed in greater detail herein, the determination of the methylation status of each of the panel of genes may be carried out in a single reaction. Many suitable techniques allowing multiplexing are available and may be utilised in the present invention. Most depend upon use of suitable fluorescent molecules having distinguishable emission spectra. The skilled person can readily select from the

many fluorophores available to determine which can be used in a multiplexing context.

In one embodiment, a universal quencher is utilised together with suitable fluorophore donors each having a distinguishable emission wavelength maximum. One suitable quencher is DABCYL. Together with a suitable quencher such as DABCYL the following fluorophores may each be utilised to allow multiplexing: Coumarin (emission maximum of 475nm), EDANS (491nm), fluorescein (515nm), Lucifer yellow (523nm), BODIPY (525nm), Eosine (543nm), tetramethylrhodamine (575nm) and texas red (615nm) (Tyagi et al., Nature Biotechnology, Vol. 16, Jan 1998; 49-53).

As discussed above, the methylation status of additional genes may also be determined in order to supplement the methods of the invention. Other genes involved in the establishment of colorectal cancer may be selected from the group consisting of CHFR, MGMT, p16, Vimentin, p14, RASSF1a, RAB32, SEPTIN-9, RASSF2A, ALX4 and SMARCA3.

The final step of the methods of the invention involve amplifying the treated isolated DNA in order to determine the methylation status of at least one gene. As discussed above, this amplification utilises 0.07 to 0.72 ml blood plasma or serum sample, equivalent of DNA per amplification reaction. Any suitable amplification technique may be utilised. In a specific embodiment, the amplifying step comprises, consists essentially of or consists of the polymerase chain reaction (PCR). It should be noted that whilst PCR is a preferred amplification method, to include variants on the basic technique such as nested PCR,

equivalents may also be included within the scope of the invention. Examples include without limitation isothermal amplification techniques such as NASBA, 3SR, TMA and triamplification, all of which are well known in the art and commercially available. Other suitable amplification methods without limitation include the ligase chain reaction (LCR) (Barringer et al, 1990), MLPA, selective amplification of target polynucleotide sequences (US Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (US Patent No 4,437,975), invader technology (Third Wave Technologies, Madison, WI), strand displacement technology, arbitrarily primed polymerase chain reaction (WO90/06995) and nick displacement amplification (WO2004/067726).

Various amplification based assays for determining the methylation status of at least one gene are known in the art, and can be used in conjunction with the present invention. These assays (including techniques such as methylation specific PCR) are described in greater detail herein, which description applies here mutatis mutandis and is not repeated simply for reasons of conciseness.

In specific embodiments, the methods of the invention employ or rely upon or utilise primers and/or probes selected from the primers and probes comprising the nucleotide sequences set forth in the relevant tables above (such as tables 2 to 18 and in particular tables 4 to 10) to determine the methylation status of the at least one gene. The tables present specific primer and probe combinations for certain preferred genes whose methylation status may be determined according to the methods of the invention.

Sequence variation that reflects the methylation status at CpG dinucleotides in the original genomic DNA offers two approaches to primer design. Both primer types may be utilised in the methods of the invention as discussed in detail herein, which discussion applies mutatis mutandis here. Suitable probes may also be employed, as described herein.

When determining methylation status, it may be beneficial to include suitable controls in order to ensure the method chosen to assess this parameter is working correctly and reliably. Suitable (positive and negative) controls are discussed in detail herein, which discussion applies mutatis mutandis.

As can be derived from the discussion and examples herein, the methylation status of the at least one gene may be correlated with the incidence of a disease for specific genes and specific diseases. Accordingly, the methods of the invention may be used in order to detect a predisposition to, or the incidence of, any disease for which gene methylation plays a role. In a specific embodiment, the disease comprises a cell proliferative disorder, although in principle any disease may be diagnosed according to these methods provided that gene methylation can be determined in an appropriate blood plasma or serum sample. The cell proliferative disorder may comprise, consist essentially of or consist of cancer for example. In particular, the cancer may comprise, consist essentially of or consist of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and

in particular colorectal cancer for example. Further specific gastrointestinal cancers are discussed above and each may be applicable to the present methods. As discussed herein, the methods may have particular application to early stage colorectal cancer, such as stage 0 to II colorectal cancer.

The invention also provides kits which may be used in order to carry out the methods of the invention. The kits may incorporate any of the various features, aspects and embodiments mentioned in connection with the various methods (and uses) of the invention above.

Thus, a kit is provided for:

(a) predicting the likelihood of successful treatment of cancer (as defined herein) and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and/or the likelihood of resistance to treatment of cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer with a DNA damaging agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor, and/or

(b) selecting a suitable treatment regimen for cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and/or

(c) diagnosing cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto, and/or

(d) determining the histopathological stage of cancer and in particular a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer or a predisposition thereto in a sample comprising carrier means containing therein a set of primers for use in detecting the methylation status of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein, in particular with respect to the methods of the invention). For example in one specific embodiment, the kit comprises carrier means containing therein a set of primers for use in detecting the methylation status of at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC. Any of the NDRG2/NDRG4-family genes may be assessed using the kits of the invention. A more detailed discussion of family members is provided above.

Thus, the kit may include suitable primers for determining whether the NDRG2/NDRG4-family gene and preferably the NDRG4 and/or NDRG2 gene is methylated. These primers may comprise any of the primers discussed in detail in respect of the various methods of the invention which may be employed in order to determine the methylation status of the NDRG2/NDRG4-family gene and preferably the NDRG4 and/or NDRG2 gene. Thus, the primers in the kit may comprise, consist essentially of, or consist of primers for the purposes of amplifying methylated or unmethylated DNA (following bisulphite treatment). In one embodiment, the primers in the kit comprise, consist essentially of, or consist of primers which are capable of amplifying methylated and/or unmethylated DNA following bisulfite

treatment which DNA comprises, consists essentially of, or consists of the nucleotide sequence set forth as SEQ ID NO: 524 and/or SEQ ID NO: 525.

The kit may alternatively or additionally employ bisulphite sequencing in order to determine the methylation status the NDRG2/NDRG4-family gene and in particular the NDRG4 and/or NDRG2 gene. Thus, the kit may comprise primers for use in sequencing through the important CpG islands in the NDRG2/NDRG4-family gene, in particular the NDRG4 and/or NDRG2 gene. Thus, primers may be designed in both the sense and antisense orientation to direct sequencing across the promoter region of the gene. In one embodiment, the primers in the kit comprise, consist essentially of, or consist of primers which are capable of sequencing of DNA following bisulfite treatment which DNA comprises, consists essentially of, or consists of the nucleotide sequence set forth as SEQ ID NO: 524 and/or SEQ ID NO: 525. Suitable primers are discussed herein in greater detail.

Similarly, the invention provides a kit for detecting a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, and MGMT, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein)

(b) means for processing a faecal sample.

As discussed in more detail above, the at least one genemay be selected from GATA4, OSMR, NDRG4 and SFRP2 since these genes provide a particularly sensitive indication of colorectal cancer. The at least one gene may be selected from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, in particular TFPI2.

The kit may comprise means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer or is used in one of the other application as discussed above. In one embodiment, the panel of genes comprises two, three, four, five or six genes.

In specific embodiments, the panel of genes comprises, consists essentially of or consists of GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2 or NDRG4 and SFRP2. In a more specific embodiment, the panel of genes comprises, consists essentially of or consists of GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2, GATA4, NDRG4 and SFRP2 or OSMR, NDRG4 and SFRP2. Further panels comprise, consist essentially of or consist of GATA4, OSMR, NDRG4 and SFRP2.

An alternative panel of genes comprises, consists essentially of or consists of NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT. The skilled person would appreciate that other combinations and permutations may be formed as

appropriate, as discussed in respect of the methods of the invention.

In one embodiment, the means for processing a faecal sample comprise a sealable vessel for collection of a faecal sample. Additionally or alternatively, the means for processing a faecal sample in the kit comprises a homogenization buffer. The means for processing a faecal sample may further or alternatively comprise reagents for extraction/isolation/concentration/purification of DNA. Suitable reagents are known in the art and comprise, consist essentially of or consist of alcohols such as ethanol and isopropanol for precipitation of DNA. Salt-based precipitation may require high concentrations of salts to precipitate contaminants. The salt may comprise, consist essentially of or consist of potassium acetate and/or ammonium acetate for example. Organic solvents may also be included in the kits to extract contaminants from cell lysates. Thus, in one embodiment, the means for processing the faecal sample comprise, consist essentially of or consist of phenol, chloroform and isoamyl alcohol to extract the DNA. Suitable combinations of reagents are envisaged as appropriate.

As discussed herein, which discussion applies *mutatis mutandis*, sensitivity of detection may be improved by increasing the quantity of DNA in the sample. Accordingly, in one embodiment the means for processing a faecal sample comprises, consists essentially of or consists of primers for directing amplification of DNA in the sample. Any suitable primers which amplify the at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23,

JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 may be utilised. The primers may not discriminate between methylated and unmethylated DNA (i.e. the primer binding sites lies outside of the CpG islands) thus providing a general increase in the amount of DNA prior to determining whether the methylated form of the gene or genes is present in the sample.

Similarly, the invention provides a kit for detecting a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

(b) means for processing a blood sample or derivative thereof.

As discussed in more detail above, the at least one gene may be selected from OSMR, NDRG4 GATA5 and ADAM23 since these genes provide a particularly sensitive indication of colorectal cancer in blood samples, or derivatives thereof and in particular plasma. The at least one gene may be selected from TFPI2, BNIP3, FOXE1, SYNE1, and SOX17, in particular TFPI2.

The kit may comprise means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel

is indicative of a predisposition to, or the incidence of, colorectal cancer or is used in one of the other application as discussed above. In one embodiment, the panel of genes comprises two, three, four, five or six genes.

In one embodiment, the panel of genes comprises, consists essentially of or consists of OSMR, NDRG4, GATA5 and ADAM23. This kit may be used to diagnose early stage colorectal cancer, in particular stage 0 to II colorectal cancer.

In one embodiment, the means for processing a blood sample or derivative thereof comprises, consists essentially of or consists of a sealable vessel for collection of a blood sample. The means for processing a blood sample or derivative thereof may further or alternatively comprises consists essentially of or consists of a reagents for extraction/isolation/concentration/purification of DNA. Suitable reagents are known in the art and comprise, consist essentially of or consist of alcohols such as ethanol and isopropanol for precipitation of DNA. Salt-based precipitation may require high concentrations of salts to precipitate contaminants. The salt may comprise, consist essentially of or consist of potassium acetate and/or ammonium acetate for example. Organic solvents may also be included in the kits to extract contaminants from cell lysates. Thus, in one embodiment, the means for processing the blood sample or derivative thereof comprise, consist essentially of or consist of phenol, chloroform and isoamyl alcohol to extract the DNA. Suitable combinations of reagents are envisaged as appropriate. The means for processing a blood sample or derivative thereof may comprise, consist essentially of or consist of isopropanol,

magnetic beads or a silica-based membrane for isolating DNA. The means for processing a blood sample or derivative thereof may comprise, consist essentially of or consist of a kit as shown in table 1.

The means for processing a blood sample or derivative thereof, in particular plasma or serum sample may comprise consist essentially of or consist of one or more stabilizers. In one embodiment, stabilizers are included in the kit to be added to the blood sample, or derivative thereof. This is particularly relevant where the sample is not frozen. In one specific embodiment, where the sample is serum, stabilizers such as stabilizers selected from EDTA and/or citrate and/or heparin are included. In a further embodiment, where the sample is plasma, stabilizers such as stabilizers selected from citrate and/or heparin may be included. Antimicrobial agents such as antibiotics may be also be included in the kits of the invention prevent spoiling (of serum and plasma samples).

Similarly, the invention provides a kit for detecting a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT

(b) means for processing a tissue sample, in particular a colon, rectal or appendix sample.

The kit may comprise means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer or is used in one of the other applications as discussed above. In one embodiment, the panel of genes comprises two, three, four, five or six genes.

In specific embodiments, the panel of genes comprises, consists essentially of or consists of OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5.

These kits may also be useful in predicting the likelihood of successful treatment of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer and/or the likelihood of resistance to treatment of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer with a DNA damaging agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor, and/or selecting a suitable treatment regimen for a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer and/or determining the histopathological stage of a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer in a sample, as discussed in

respect of the methods of the invention (which discussion applies *mutatis mutandis*).

In a further embodiment, applicable to all relevant kits of the invention, the means for detecting an epigenetic change in the panel of genes enable the detection to be carried out in a single reaction. Multiplexing is made possible for example through use of appropriate fluorophores having separable emission spectra. TaqMan probes, Molecular Beacons, Scorpions, etc…, as discussed herein, allow multiple markers to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Accordingly, suitably labelled probes and primers are encapsulated by the kits of the invention.

In a particularly preferred embodiment, the epigenetic change which is detected using the kits of the invention is methylation. Many suitable reagents for methylation detection are known in the art, and are discussed herein (which discussion applies here *mutatis mutandis*). In particular, hypermethylation of the promoter region of the gene(s) may be detected using the kits of the invention. Thus, the means for detecting methylation may comprise methylation specific PCR primers. Suitable primers may be selected from the primers comprising, consisting essentially of or consisting of the nucleotide sequences presented in any one of tables 2 to 18 as appropriate depending upon the kit and gene or genes concerned.

The kit may also include means for carrying out the methylation specific PCR in real time or at end point. The

means for carrying out the methylation specific PCR/amplification in real time or at end point may comprise hairpin primers (Amplifluor), hairpin probes (Molecular Beacons), hydrolytic probes (Taqman), FRET probe pairs (Lightcycler), primers incorporating a hairpin probe (Scorpion), fluorescent dyes (SYBR Green etc.), DzyNA primers or oligonucleotide blockers for example. Suitable probes may be selected from the probes comprising, consisting essentially of or consisting of the nucleotide sequences presented in tables 2 to 18 as appropriate for the respective genes. All appropriate combinations are envisaged by the invention. Primers and probes for detecting a suitable reference gene, such as beta-actin are displayed in some of these tables (3 and 4).

The end-point PCR fluorescence detection technique can use the same approaches as widely used for Real Time PCR - TaqMan assay, Molecular Beacons, Scorpion etc. Accordingly, the kits of the invention may, in certain embodiments, include means for carrying out end-point methylation specific PCR. The means for carrying out end-point methylation specific PCR/amplification may comprise primers and/or probes as explained for PCR/amplification in Real-time.

In the real-time and end-point detection embodiments, the probes for detection of amplification products may simply be used to monitor progress of the amplification reaction in real-time and/or they may also have a role in determining the methylation status of the at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT,

TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), themselves. Thus, the probes may be designed in much the same fashion as the primers to take advantage of sequence differences following treatment with a suitable reagent such as sodium bisulphite dependent upon the methylation status of the appropriate cytosine residues (found in CpG dinucleotides).

The probes may comprise any suitable probe type for real-time detection of amplification products as discussed above. Notably, however, with the AMPLIFLUOR and SCORPION embodiments, the probes are an integral part of the primers which are utilised. The probes are typically fluorescently labelled, although other label types may be utilised as appropriate (such as mass labels or radioisotope labels). These probes are also suitable for end-point detection.

The kits of the invention may be kits for use in MSP and in particular in a real-time or end point detection version of MSP.

The kits of the invention may incorporate reagents for quantification of DNA such as those found in the Picogreen® dsDNA quantitation kit available from Molecular Probes, Invitrogen.

The kits of the invention may, additionally or alternatively comprise, consist essentially of or consist of a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated

cytosine residues. The reagent preferably comprises, consists essentially of or consists of a bisulphite reagent. The bisulphite reagent most preferably comprises, consists essentially of or consists of sodium bisulphite. This reagent is capable of converting unmethylated cytosine residues to uracil whereas methylated cytosines remain unconverted. This difference in residue may be utilised to distinguish between methylated and unmethylated nucleic acid in a downstream process, such as PCR using primers which distinguish between cytosine and uracil (cytosine pairs with guanine, whereas uracil pairs with adenine). The reagent may be incorporated as the means for processing a faecal sample or means for processing a blood sample or derivative thereof depending upon the kit in question.

As discussed with respect to the methods of the invention, suitable controls may be utilised in order to act as quality control for the methods. Accordingly, in one embodiment, the kit of the invention further comprises, consists essentially of or consists of one or more control nucleic acid molecules of which the methylation status is known. These (one or more) control nucleic acid molecules may include both nucleic acids which are known to be, or treated so as to be, methylated and/or nucleic acid molecules which are known to be, or treated so as to be, unmethylated. One example of a suitable internal reference gene, which is generally unmethylated, but may be treated so as to be methylated, is ß-actin.

Furthermore, the kit of the invention may further comprise, consist essentially of or consist of primers for the amplification of the control nucleic acid. These primers may be the same primers as those utilised to monitor

methylation in the test sample in specific embodiments. Thus, the control nucleic acid may comprise at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), for example taken from normal tissues in which it is known to be unmethylated. The control nucleic acid may additionally comprise at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein) in methylated form, for example as methylated by a methyltransferase enzyme such as SssI methyltransferase for example.

Suitable probes and/or oligonucleotide blockers for use in determining the methylation status of the control nucleic acid molecules may also be incorporated into the kits of the invention. The probes may comprise any suitable probe type for real-time detection of amplification products. The discussion provided above applies *mutatis mutandis.*

The kits of the invention may additionally include suitable buffers and other reagents for carrying out the claimed methods of the invention. Thus, the discussion provided in respect of the methods of the invention as to the requirements for determination of the methylation status of at least one gene selected from an NDRG2/NDRG4 subfamily gene (in particular NDRG4), GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1,

SOX17, PHACTR3 and JAM3 (in all permutations and combinations including panels as discussed herein), apply mutatis mutandis here.

In specific embodiments, the kit of the invention further comprises, consists essentially of, or consists of nucleic acid amplification buffers. Suitable reagents may be selected from $(NH_4)_2SO_4$, Tris (pH 8.8), $MgCl_2$, ß-mercaptoethanol and stock solutions of dNTPs. Reagents may be supplied at any suitable concentration.

The kit may also additionally comprise, consist essentially of or consist of enzymes to catalyze nucleic acid amplification. Thus, the kit may also additionally comprise, consist essentially of or consist of a suitable polymerase for nucleic acid amplification. Examples include those from both family A and family B type polymerases, such as Taq (such as the commercially available Jumpstart DNA Taq polymerase), Pfu, Vent etc.

The various components of the kit may be packaged separately in separate compartments or may, for example be stored together where appropriate.

The kit may also incorporate suitable instructions for use, which may be printed on a separate sheet or incorporated into the kit packaging for example.

In one specific aspect, the methods and kits of the invention may be combined with the other methods and kits of the invention in order to provide improved diagnosis, histopathological analysis, pharmacogenomic analysis etc. of

a gastrointestinal cancer, such as colorectal cancer and/or gastric cancer and/or oesophageal cancer and in particular colorectal cancer. Accordingly, all embodiments of the methods and kits of the invention apply *mutatis mutandis* to the respective aspects of the invention.

The invention will now be described with respect to the following non-limiting examples.

DETAILED DESCRIPTION OF THE INVENTION

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1.

a. NDRG4: Bisulfite sequencing of colorectal cancer tissue (T), normal colon mucosa (N), the methylated colorectal cancer cell line (HCT116) and the unmethylated cell line SW480. White and black squares represent methylated and unmethylated CpG dinucleotides in NDRG4 respectively. Each row represents a single clone. Location of the CpG are relative to the transcription start site. The location of the MSP primers is positions 20 to 23 and 31 to 34 respectively.

b. NDRG2: Bisulfite sequencing of colon carcinoma cell lines (RKO and LS174T). White and black squares represent methylated and unmethylated CpG dinucleotides in NDRG2B respectively.

FIG. 2. Relative expression of NDRG4 after treatment with DAC and TSA compared to untreated cell lines. Cyclophilin was used as a reference gene for expression normalisation.

FIG. 3. a. Methylated NDRG4 sequence (SEQ ID NO: 524)(NM_020465: -1000 to +1000 relative to TSS) Bisulfite sequence primers in mid-grey; Flank primers for the nested MSP are underlined; Methylated MSP primers in light-grey; Unmethylated primers in dark-grey and light-grey

FIG. 3. b. Methylated NDRG2 sequence (SEQ ID NO: 525). Bisulfite sequence primers in mid-grey; Flank primers for the nested MSP are underlined; Methylated MSP primers in light-grey; Unmethylated primers in dark-grey and light-grey

FIG. 4. Sentivity of different markers, with 100% specificity. X axis=: % positive in real time QMSP; Y axis=: different markers. Case: n = 65 carcinoma's; Controls: n = 33 histologically normal resection ends
FIG. 5 shows a decision tree for determination of the methylation status of the gene of interest linked to colorectal cancer in clinical samples (real-time MSP).

FIG. 6 presents results of real-time MSP carried out on 9 different genes for 34 colon carcinoma tissue samples, 16 colon adenoma tissue samples and 63 breast (20), lung (21) and bladder (22) cancer samples. Sensitivity performance for each gene is shown wherein the analytical cut-off was set to give 100% specificity (based on the non-cancerous controls).

FIG. 7. Presents results of real-time MSP carried out on 10 different genes for 34 colon carcinoma tissue samples, 16 colon adenoma tissue samples and 59 samples from patients with cancer other than CRC. Sensitivity performance for

each gene is shown wherein the analytical cut-off was set to give 100% specificity (based on the non-cancerous controls), except for JPH3 where 95% specificity was obtained.

FIG. 8. Presents results of real-time MSP carried out on 8 different genes for plasma training set 1 and 5 different genes for plasma training set 2. Plasma training set 1 includes 34 samples with no suspicious findings, 25 samples from patients with cancers other than colon and 42 samples from patients covering all stages of CRC, with 81% representing stages I-III of disease. Plasma training set 2 was tested on 64 samples with no suspicious findings, 49 adenomas, 25 samples from patients with cancer other than colon cancer and 78 samples from patients covering all stages of CRC, with 76% representing stages I-III of disease.

FIG. 9. Is an overview of the NDRG4 study showing the patient groups which were investigated.

FIG. 10. Schematic representation of the promoter region of NDRG4. A dense CpG island from -556 to +869 relative to the transcription start site (TSS) (indicated by a curved arrow) is shown. Locations of CpG dinucleotides (representated by |), ORF NDRG4 (as indicated with a grey rectangle) and the region of the hypermethylated fragment identified by Methylation Specific PCR (MSP), Quantative MSP (qMSP) and Bisulfite sequencing (BS) primers are indicated.

FIG. 11a. Results of methylation specific PCR (MSP) with primer pair 2 to detect DNA methylation in eight different CRC cell lines.

FIG.11b Bisulfite sequencing of two CRC cell lines, namely HCT116 and SW480. Six different clones were sequenced. Each row represents an individual cloned allele that was sequenced following sodium bisulfite DNA modification. Each box indicate a CpG dinucleotide (black box; methylated CpG site, white box; unmethylated CpG site)

FIG. 11c NDRG4 expression in colon cancer cell lines (RKO and HCT116) after treatment with the methylation inhibitor 5-aza-2'-doxycytidine (DAC).

FIG. 12a. Bisulfite sequencing of three cases of cancers (T) and their matched normal non malignant mucosa tissue (N). Six different clones were sequenced.

FIG. 12b Levels of NDRG4 transcript expression measured by realtime PCR in colon cancer tissue (labelled for T) and matched normal colon tissue samples (labelled for N) for three different persons. For each patient, levels of NDRG4 expression in the normal mucosa tissue were set to equal 1. The experiments were performed three times.

FIG. 12c Localization of NDRG4 expression. Immunohistochemical staining of NDRG4 in normal mucosa and colon tumor shows no staining in cancer cells but clear staining in the nuclei of normal epithelial cells.

EXPERIMENTAL SECTION

1) NDRG EXPERIMENTS

Cell culture

Colon cancer cell lines LS174T, HCT116, HT29, RKO, CaCo2, Colo205, SW48 and SW480 were used for MSP, bisulfite sequencing and real time (reexpression) RT-PCR (1 MM DAC and 300 nM TSA).

Study population

Formalin-fixed, paraffin-embedded colon mucosa tissue of colorectal cancer patients and controls over 50 years of age was retrospectively collected from the archive of the dept. of Pathology of the University Hospital Maastricht. Approval was obtained by the Medical Ethical Committee (MEC) of the Maastricht University and the University Hospital Maastricht. If present, also normal and adenoma tissue was collected from these cases. The control group consists of histologically normal biopsy material from patients undergoing endoscopy because of non-specific abdominal complaints, adenoma biopsies from patients which did not develop colorectal cancers within 5-10 years. Colorectal cancers patients and controls were excluded if being diagnosed with additional cancers other than non-melanoma skin cancer.

Methylation-Specific PCR

DNA methylation in the CpG islands of the gene promoter was determined by bisulfite treatment of genomic DNA with sodium bisulfite followed by MSP. Briefly, bisulfite modification of genomic DNA was carried using the EZ DNA methylation kit (Zymo Research). MSP analysis on DNA retrieved from formalin-fixed, paraffin embedded tissue was facilitated by first amplifying the DNA with flanking PCR primers which amplify bisulfite-modified DNA but do not make the

distinction between methylated or unmethylated DNA. This PCR product was used as a template for the MSP reaction. All PCRs were performed with controls for unmethylated DNA (DNA from normal lymfocytes), methylated DNA (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)), and a control without DNA. Ten µl of each MSP reaction were directly loaded onto 2% agarose visualized under UV illumination. Primer sequences and PCR conditions, are specified in Table 19.

Alternatively, DNA methylation was determined by QMSP.

Table 19: NDRG4 and NDRG2b MSP primers

SEQ ID No	Gene	Primer name	Sequence 5'-3	Size	Ann. Temp.	Cycles	Posn.*
5	NDRG4	Flank F	ggttygttygggattagttttagg	155 bp	56	35	-144
6	NDRG4	Flank R	craacaaccaaaaacccctc				+10
7	NDRG4	U sense	gattagttttaggtttggtattgttttgt	100 bp	66	25	-133
8	NDRG4	U antisense	aaaaccaaactaaaaacaatacacca				-34
9	NDRG4	M sense	tttaggttcggtatcgtttcgc	88 bp	66	25	-126
10	NDRG4	M antisense	cgaactaaaaacgatacgccg				-39
20	NDRG2	Flank F	YGTTTTTTATTTATAGYGGTTTTT				
21	NDRG2	Flank R	TCCTAATACCTCTCCTCTCTTTACTAC				
22	NDRG2	U sense	TTTTATTTATAGTGGTTTTTTGTATTTTTT				
23	NDRG2	U antisense	TCTCCTCTCTTTACTACATCCCAACA				
24	NDRG2	M sense	TTTATAGCGGTTTTTCGTATTTTTC				
25	NDRG2	M antisense	CCTCTCTTTACTACGTCCCGACG				

* Position relative to transcription start site

Bisulfite genomic sequencing

Genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Leiden, the Netherlands).

Bisulfite modification of genomic DNA was carried out using the EZ DNA methylation kit (Zymo Research). PCR products were subcloned using the TA cloning kit (Invitrogen, Breda, the Netherlands) and single colonies were selected and sequenced. Primer sequences and PCR conditions are specified in Table 20.

Table 20: NDRG4 and NDRG2b bisulfite sequencing primers

SEQ ID NO	Gene	Primer name	Sequence 5'-3	Size	Ann. Temp.	Cycles	Position
570	NDRG4	F	gatyggggtgttttttaggttt	262 bp	64	40	-251
6	NDRG4	R	craacaaccaaaaaccccctc				+10
522	NDRG2	F	TTTGTTGGTTATTTTTTTTTTATTTTT				
523	NDRG2	R	CCCCCAAACTCAATAATAAAAAC				

Real-time RT-PCR

Total RNA isolation was isolated by use of the Rneasy Mini kit (Qiagen) cDNA synthesis using the Iscript cDNA synthesis kit (Bio-Rad). Quantitative real-time reverse transcription-PCR was done using SYBR Green PCR Master Mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Primers and PCR conditions are specified in Table 21.

Table 21: NDRG4 Real time RT-PCR primers

SEQ ID NO	Gene	Primer name	Sequence 5'-3	Size	Ann. Temp.	Cycles
1	NDRG4	F	cctgaggagaagccgctg	101bp	60	40
2	NDRG4	R	atgtcatgttccttccagtctgt			

Expression analysis of NDRG4

Expression of the NDRG4 gene was determined by real-time reverse-transcription PCR (RT-PCR). The NDRG4 gene was found to be well expressed in normal colon cell lines, whereas it

was not expressed in the colon cancer cell lines. Since this on its own did not indicate that the silencing is epigenetic, the RKO and HCT116 cell lines were treated with the reagent DAC (5'dazacytidine) and TSA. Relative expression of NDRG4 after treatment with DAC and TSA was compared to untreated cell lines. Cyclophilin was used as a reference gene for expression normalisation. FIG 2 shows that treatment resulted in a reactivation of NDRG4 expression, providing evidence for epigenetic silencing of the gene in colon cancer cells.

CpG island methylation status analysis of NDRG4 and NDRG2

Having observed that the silencing of NDRG4 expression was reversed after treatment with DAC and TSA, the association between the transcriptional inactivation and the putative epigenetic aberration was further investigated. The NDRG CpG island methylation status was established by PCR analysis of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil, using the procedures as specified. Table V shows that NDRG4 CpG island methylation analysed by MSP was observed in the cancer cell lines LS174T, HCT116, HT29, RKO, CaCO2 and SW48, whereas it was absent in the unmethylated cell line SW480.
Similarly, NDRG2 CpG island methylation analysed by MSP with different primer sets (a to d) was observed in most of the cancer cell lines. In all cancer cell lines LS174T, HCT116, HT29, RKO, CaCO2 and SW48, NDRG2 CpG island methylation was observed with primer sets b of table 19.

Table 22: Methylation status of colorectal cancer cell lines (analysed by MSP)

	LS174T	HCT116	HT29	RKO	CaC02	Colo205	SW48	SW480
NDRG4								U
NDRG2a	U	U	U	U	U	U	U	/
NDRG2b	U		U					/
NDRG2c	U		U					U?
NDRG2d(a)	U		U			U	U	M

Following the demonstration of the epigenetic loss of function of NDRG4 in cancer-cell lines, we assessed the prevalence of NDRG4 CpG island promoter hypermethylation in cancer patients. As expected, NDRG4 CpG island promoter hypermethylation was absent in normal mucosa from patients without cancer. As indicated in Table 23, NDRG4 CpG island promoter hypermethylation was observed with different frequency among each class of neoplasm. NDRG4 was methylated in 76% of the 88 investigated carcinoma tissues and in 57% of 57 adenomas with concurrent colorectal cancer. In adenomas from patients that did not have colorectal cancer (low-grade dysplastic non-progressed adenomas), NDRG4 methylation was significantly lower (14%), indicating the prognostic value of this NDRG4 methylation towards colorectal cancer development

Table 23: Prevalence of NDRG4 methylation in colorectal tissue

	Methylation (%)
Morphologically normal mucosa adjacent to tumor tissue	2.5 (n=82)
Adenomas from patients also presenting a colorectal carcinoma	57 (n=57)
Carcinoma tissue	76 (n=88)

Normal mucosa from patients without cancer	0 (n=27)
Adenomas from patients that did not develop colorectal cancer (low-grade dysplastic non-progressed adenomas)	14 (n=51)

NDRG4 methylation compared to methylation of other markers

Samples from resected tumors and histologically normal resection were tested for hypermethylation of 13 genes. Representative results are shown in FIG 4. The highest methylation was obtained for SFRP1, SFRP2, NDRG4, GATA4 and GATA5. All showed a sensitivity >40% for 100% specificity. We tested the ability of the NDRG4 methylation marker to improve the sensitivity of cancer detection with a number of methylation markers selected on their ability to detect colorectal cancer. The other genes were selected from the group consisting of SFRP1, SFRP2, GATA-4, GATA-5, CHFR, APC(2), MGMT, p16, Vimentin, p14, RASSF1a and RAB32. In a first instance, the ability of NDRG4 to complement SFRP1 was analysed. 30% of colon carcinoma samples (n=18) for which SFRP1 failed to be hypermethylated, showed hypermethylation for NDRG4 (n=6). Similarly, carcinoma samples which failed to be detected by way of SFRP2, GATA4, or GATA5 methylation analysis, showed hypermethylation for NDRG4. In fact, the combination of NDRG4 with any of the methylation markers from FIG 4 improved diagnosis of cancer

NDRG-4 MSP on other cancer types (methylated cancers)

NDRG-4 methylation was assessed on other cancer types showing hypermethylation for certain genes. These cancer types comprised melanoma, clear cell kidney cancer, ovarian carcinoma, prostate cancer, breast cancer and gastric cancer. The results were as follows:

Melanoma: 0 out of 8 samples were methylated for NDRG4

Clear cell kidney cancer: only 1 out of 10 samples was methylated for NDRG4

Ovarium carcinoma: 0 out of 20 samples were methylated for NDRG4

Prostate cancer: 0 out of 10 samples were methylated for NDRG4

Breast cancer: 0/7 lobular cancers and 0/9 Ductal cancers were methylated for NDRG4

In contrast to these results, in all of the 6 gastric cancers tested methylation for NDRG4 was observed. This seems to indicate that NDRG4 is a type-specific cancer methylation marker and is preferably used to detect colon cancer and/or gastric cancer.

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2) EXPERIMENTS ON FAECAL DNA

Example 1

MATERIALS AND METHODS IN RELATION TO FAECAL DNA

Sample collection and processing

A standardized multicenter screening trial (The Netherlands) was initiated in 2006. In this trial, non symptomatic subjects aged 50 or above are screened with colonoscopy, FOBT and real-time MSP using DNA from stool and blood. In addition, prospectively collected stool samples from multiple centers (Germany and The Netherlands) were used. In these trials, symptomatic patients, attending a Gastroenterology clinic and ultimately diagnosed with CRC, provided a stool sample for use in real-time MSP. From the ongoing trials 147 stool samples were available for the present study. 3 main categories of stool samples were

used: 67 samples with no suspicious findings, 58 adenomas and 22 samples from patients covering all stages of CRC, with 90% representing early stage disease.

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

DNA extraction from stool

Single aliquots (32 ml containing the equivalent of 4 g of stool) were centrifuged for 5 minutes at 2540 rcf at 20°C. The supernatant was retained and centrifuged a second time (10 minutes at 16500 rcf at 4°C). 22 ml of the supernatant obtained following the second centrifugation step was incubated with 5 µl Rnase A for 60 minutes at 37°C. Total DNA was then SodiumAcetate (pH 5.2) - isopropanol precipitated and washed with 70% ethanol. The DNA was resuspended in 4 ml 1x TE (pH 7.4). 400 µl 10x buffer (240 mM EDTA (pH=8.0), 750 mM NaC), 400 µl 10% SDS, and 20 µl Proteinase K (20 mg/ml) was added and the samples were incubated at 48°C overnight at constant shaking (225 RPM). After centrifugation (3000 RCF for 30 seconds at room temperature), 5 ml of Phenol: Chloroform:Isoamylalcohol (25:24:1, v/v; Invitrogen) was added and incubated for 10 minutes at room temperature shaking at 225 RPM and centrifuged for 5 minutes at 3000 RCF. The aqueous layer was transferred to a new tube containing 5 ml of Phenol: Chloroform:Isoamylalcohol. Again, the samples were incubated for 10 minutes at room temperature shaking at 225 RPM and

centrifuged for 5 minutes at 3000 RCF at room temperature. The aqueous layer was transferred to a new tube and DNA was precipitated by adding 500 µl 7.5 M Ammonium Acetate, 5 µl glycogen and 10 ml of cold 100% Ethanol (-20°C), further incubated at -20°C for at least 1 hour and centrifuged at 15000 RCF for 30 minutes at 4°C. Pellets were washed with 3.5 ml freshly prepared 70% Ethanol and air dried. Pellets were finaly resuspended in 2 ml of LoTE pH 8.0 and stored at -80°C, until further processing. Average yield of DNA was 462 µg (ranging from 46 - 2127 µg; SD 420)

DNA modification

An upscaled DNA modification step was applied to 32 µg of the obtained DNA. 16 Aliquots of 2 µg of DNA were subjected to bisulfite modification in 96-wells format on a pipetting robot (Tecan), using the EZ-96DNA Methylation kit (Zymo Research), according to the manufacturer's protocol. Basically, aliquots of 45 µl were mixed with 5 µl of M-Dilution Buffer and incubated at 37°C for 15 minutes shaking at 1100 rpm. Then 100 µl of the diluted CT Conversion Reagent was added and samples were incubated at 70°C for 3 hours, shaking at 1100 rpm in the dark. After conversion, the samples were desalted by incubation on ice for 10 minutes and addition of 400 µl of M-Binding buffer. The samples were loaded on a Zymo-Spin I Column in a collection tube and after centrifugation washed with 200 µl of M-Wash Buffer. 200 µl of M-Desulphonation Buffer was put onto the column and incubated at room temperature for 15 minutes. After centrifugation of the columns, they were washed twice with 200 µl of M-Wash Buffer. Finally, the DNA was washed from the column in 50 µl Tris-HCl 1mM pH8.0 and stored at -80°C, until further processing.

DNA concentration

Bisulfite treated DNA is concentrated using the ZYMO Clean and Concentrator Kit (Zymo Research). To each aliquot of DNA 100 µl of DNA Binding Buffer was added. The equivalent of ~6 µg of DNA (quantified before bisulfite treatment) was transferred to a Zymo-Spin™ Column in a collection tube. (16 wells with bisulfite treated DNA per sample are divided over 5 Zymo-SpinTM columns.) The tubes were centrifuged at ≥10,000 rpm for 30 seconds and washed twice with 200 µl of wash buffer. The DNA was eluted of the column by adding 6 µl of 1 mM Tris-HCl, pH=8.0, incubated for 1 minute and centrifugation at ≥10,000 rpm for 30 seconds. The eluates of columns with the same sample were pooled. The resulting chemical treated DNA was used as template for real-time MSP.

DNA amplification

Real-time MSP was applied on a 7900HT fast real-time PCR system (Applied Biosystems). 2.4 µl of the modified DNA (equivalent to 2,5 µg unconverted DNA) was added to a PCR mix (total volume 12 µl) containing buffer (16.6mM (NH4)2SO4, 67 mM Tris (pH 8.8), 6.7 mM MgCl2, 10 mM ß-mercaptoethanol), dNTPs (5 mM), forward primer (6 ng), reverse primer (18 ng), molecular beacon (0.16 µM), BSA (0.1 µg), and Jumpstart DNA Taq polymerase (0.4 units; Sigma Aldrich). The primer sequences and molecular beacon sequences used for each of the genes are summarized in table 1. Cycle program used was as follows: 5 minutes 95°C, followed by 45 cycles of 30 seconds 95°C, 30 seconds 57°C (51°C for APC), and 30 seconds 72°C, followed by 5 minutes 72°C. A standard curve (2x106 - 20 copies) was included to

determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

RESULTS

Marker identification and validation in colon tissue samples.

Assay validity rate in tissue and stool: 230 FFPE and 147 stool samples were processed using real-time MSP. The real-time MSP assays produced valid results in 99% of the FFPE and stool samples.

Marker selection in colon tissue: Based on re-expression, 224 different gene assays representing 145 gene promotors were tested on the Base5 methylation profiling platform (data not shown, see reference 1 for details). The 37 most differentially methylated gene sequences assessing 29 gene promoters were validated on retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends (histopathologically normal) using real-time MSP. Several markers reliably detected CRC in those tissue samples (data not shown). The results were confirmed on an independent test set containing 39 tissue controls (non-cancerous), 34 carcinomas and 16 adenomas. Several combinations of the tested markers reliably detected CRC with high specificity and sensitivity.

The ten best performing markers GATA5, GATA4, SFRP1, SFRP2, APC, MGMT, NDRG4, OSMR, JPH3 and ADAM23 were validated with primer sets and beacon probes as specified in Table 24. In addition to the colon test genes, the independent reference gene ß-Actin (ACT) was also measured. The ratios between

the colon test genes and ACT were calculated, and are the test result of the assay. The samples were classified as methylated, non-methylated, or invalid based on the decision tree shown in FIG. 5.

The individual performance of the ten markers is shown in Table 25. Dependent on the cutoff applied, different sentivities were obtained for the individual markers. For 100% specificity of the marker, sensitivities (%) ranged from 56 to 66 for GATA5, 78 to 82 GATA4, 84 to 92 for SFRP1, 72 to 84 for SFRP2, 40 to 46 for APC, 44 for MGMT, 64 to 66 for NDRG4, 88 for OSMR, 82 for JPH3 and 50 for ADAM23.

Table 24: Primers sequences and beacon sequences

SEQ ID NO:			
26	ß-Actin	forward primer	5' - TAGGGAGTATATAGGTTGGGGAAGTT - 3'
27		reverse primer	5' - AACACACAATAACAAACACAAATTCAC - 3'
28		beacon	5'-FAM-CGACTGCGTGTGGGGTGGTGATGGAGGAGGTTTAGGCAGTCG-3'-DABCYL
29	GATA4	forward primer	5' - AGGTTAGTTAGCGTTTTAGGGTC - 3'
30		reverse primer	5' - ACGACGACGAAACCTCTCG - 3'
31		beacon	5'-FAM-CGACATGCCTCGCGACTCGAATCCCCGACCCAGCATGTCG-3'-DABCYL
32	GATA5	forward primer	5' - AGTTCGTTTTTAGGTTAGTTTTCGGC - 3'
33		reverse primer	5' - CCAATACAACTAAACGAACGAACCG - 3'
34		beacon	5'-FAM-CGACATGCGTAGGGAGGTAGAGGGTTCGGGATTCGTAGCATGTCG-3'-DABCYL
35	SFRP1	forward primer	5' -TGTAGTTTTCGGAGTTAGTGTCGCGC- 3
36		reverse primer	5' -CCTACGATCGAAAACGACGCGAACG- 3'
37		beacon	5'-FAM-CGACATGCTCGGGAGTCGGGGCGTATTTAGTTCGTAGCGGCATGTCG-3'-DABCYL
38	SFRP2	forward primer	5' - GGGTCGGAGTTTTTCGGAGTTGCGC - 3'
39		reverse primer	5' - CCGCTCTCTTCGCTAAATACGACTCG - 3'
40		beacon	5'-FAM-CGACATGCGGTGTTTCGTTTTTTCGCGTTTTAGTCGTCGGGCATGTCG -3'-DABCYL
17	NDRG4	forward primer	5' - GTATTTTAGTCGCGTAGAAGGC - 3'
18		reverse primer	5' - AATTTAACGAATATAAACGCTCGAC - 3'
19		beacon	5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL
41	APC	forward primer	5'-GAACCAAAACGCTCCCCAT-3'

42		reverse primer	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
43		beacon	5' -FAM-CGTCTGCCCCGTCGAAAACCCGCCGATTAACGCAGACG-3'-DABCYL
44	ADAM23	forward primer	5' - GAAGGACGAGAAGTAGGCG - 3'
45		reverse primer	5' - CTAACGAACTACAACCTTACCGA - 3'
46		beacon	5'-FAM-CGACATGCCCCCGACCCGCACGCCGCCCTGCATGTCG-3'-DABCYL
47	OSMR (3)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
48		reverse primer	5' - CGAACTTTACGAACGAACGAAC - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
47	OSMR (4)	forward primer	5' - TTTGGTCGGGGTAGGAGTAGC - 3'
50		reverse primer	5' - AAAAACTTAAAAACCGAAAACTCG - 3'
49		beacon	5'-FAM-CGACATGCCCGTACCCCGCGCGCAGCATGTCG-3'-DABCYL
51	JPH3	forward primer	5' - TTAGATTTCGTAAACGGTGAAAAC - 3'
52		reverse primer	5' - TCTCCTCCGAAAAACGCTC - 3'
53		beacon	5'-FAM-CGTCTGCAACCGCCGACGACCGCGACGCAGACG-3'-DABCYL
54	MGMT	forward primer	5' - TTTCGACGTTCGTAGGTTTTCGC - 3'
55		reverse primer	5' - GCACTCTTCCGAAAACGAAACG - 3'
56		beacon	5'-FAM-CGTCTCGCGTGCGTATCGTTTGCGATTTGGTGAGTGTTTGGGGCGAGACG-3'-DABCYL

Table 25: Individual performance of markers on adenoma and carcinoma colorectal tissue samples

Gene *	Cases (adenoma+carcinoma)	Controls	Cut off ratio**	Sensitivity (%)	Specificity (%)
GATA5	50	39	12 (5)	56 (66)	100
GATA4	50	39	17 (12)	78 (82)	100
SFRP1	50	39	47 (25)	84 (92)	100
SFRP2	50	39	28 (9)	72 (84)	100
APC	50	39	16 (5)	40 (46)	100
MGMT	50	39	18	44	100
NDRG4	50	39	7 (1)	64 (66)	100
OSMR (3)	50	39	47	88	100
JPH3	50	39	55 (75)	82 (82)	95 (100)
ADAM23	50	39	2	50	100

* (3) reflects the primer combinations used for assessing methylation of the OSMR gene

** In case two sets of cut off ratio were assessed, the second set and its corresponding sensitivity is indicated between ( ).

Complementarity of markers

The different markers were tested on their complementarity. Several combinations of the tested markers reliably detected CRC with high specificity and sensitivity. Results are summarized in Table 26. For 100% specificity, sensitivities (%) ranged between 90 to 98 for combinations of two markers. A sensitivity of 100% was obtained for the 3-marker combinations SFRP1+SFRP2+APC and SFRP2+OSMR+APC.

Table 26: Complementarity of markers on adenoma and carcinoma colorectal tissue samples

Genes *	Sensitivity **	Specificity
NDRG4 + OSMR (4)	90%	100%
SFRP2 + APC	92%	100%
APC + OSMR (3)	92%	100%
MGMT + OSMR (3)	92%	100%
OSMR (3) + OSMR (4)	92%	100%
SFRP1 + APC	94%	100%
SFRP1 + GATA-4	94%	100%
SFRP1 + NDRG4	94%	100%
SFRP1 + OSMR (3)	94%	100%
SFRP1 + OSMR (4)	94%	100%
GATA-4 + OSMR (4)	94%	100%
NDRG4 + OSMR (3)	94%	100%
GATA-5 + SFRP1	96%	100%
GATA-5 + OSMR (3)	96%	100%
SFRP2 + OSMR (4)	96%	100%
GATA-4 + OSMR (3)	96%	100%
SFRP1 + SFRP2	98%	100%
SFRP2 + OSMR (3)	98%	100%
SFRP1 + SFRP2 + APC	100%	100%
SFRP2 + OSMR (3) + APC	100%	100%

* (3) and (4) reflect the primer combinations used for assessing methylation of the OSMR gene

** Sensitivity corresponding to the second cutoff set specified between () in Table 25.

Performance of markers on adenoma and carcinoma tissue samples

Important for early cancer detection is the performance of the markers on early stage cancers. Therefore, the 50 cancer cases from the test set were further divided into 2 diagnosis groups: carcinomas and adenomas. Results are summarized in table 27 and 28. Sensitivity for carcinomas ranged from 35% to 88% for detection of colorectal cancer whereas sensitivity for adenomas ranged from 31% to 88% both with a corresponding specificity of 100%. These results indicate that the selected set of genes are highly specific for colorectal cancer and include some promising early stage detection markers.

Table 27: Performance of the markers on carcinoma samples

Gene *	Carcinoma	Controls	Cut off ratio	Sensitivity	Specificity
GATA5	34	39	12	53	100
GATA4	34	39	17	74	100
SFRP1	34	39	47	82	100
SFRP2	34	39	28	68	100
APC	34	39	16	35	100
MGMT	34	39	18	35	100
NDRG4	34	39	7	62	100
OSMR (3)	34	39	47	88	100
JPH3	34	39	55	82	100
ADAM23	34	39	2	59	100

* (3) reflects the primer combinations used for assessing methylation of the OSMR gene

Table 28: Performance of the markers on adenoma samples

Gene *	adenoma	Controls	Cut off ratio	Sensitivity	Specificity
GATA5	16	39	12	63	100
GATA4	16	39	17	88	100

Gene *	adenoma	Controls	Cut off ratio	Sensitivity	Specificity
SFRP1	16	39	47	88	100
SFRP2	16	39	28	81	100
APC	16	39	16	50	100
MGMT	16	39	18	63	100
NDRG4	16	39	7	69	100
OSMR (3)	16	39	47	88	100
JPH3	16	39	55	81	100
ADAM23	16	39	2	31	100

* (3) reflects the primer combinations used for assessing methylation of the OSMR gene

Performance of markers in fecal samples

Nine of the best performing methylation markers in tissue (GATA4, GATA5, SFRP1, SFRP2, NDRG4, APC, ADAM23, OSMR3, and JPH3) were chosen to be evaluated in fecal samples. ß-Actin copy numbers were also quantified as a control for sample quality and DNA yield.

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

The individual performance of the 9 genes (Actin, SFRP2, GATA5, GATA4, APC, SFRP1, NDRG4, OSMR3 and ADAM23) in fecal samples from adenoma's and colorectal cancers is shown in Table 29. A specificity of 100% was obtained for most of the genes, except for SFRP2. The best performing genes in fecal samples from patients with CRC corresponded to GATA4 with 73% sensitivity, SFRP1 with 67% sensitivity, OSMR3 with 67% sensitivity, and NDRG4 with 60% sensitivity, all with a corresponding specificity of 100%.

Table 29: Performance of the markers in fecal samples

	Number	Act	SFRP2	GATA5	GATA4	APC	SFRP1	NDRG4	OSMR (3)	Adam23
cutoff (copies)	of samples	200	1	1	4	1	1	0	10	1

Sens adenoma	13	15%	38%	0%	15%	8%	8%	15%	8%	0%
Sens CRC	15	67%	67%	27%	73%	47%	67%	60%	67%	40%
Spec	19	95%	84%	100%	100%	100%	100%	100%	100%	100%

Performance of marker combination panels in fecal samples

Four candidate methylation markers were found to result in the best sensitivity and specificity in stool samples: GATA4, SFRP2, NDRG4, OSMR. ß-Actin copy numbers were also quantified as a control for sample quality and DNA yield. The performance of combination panels of these 4 methylation markers was investigated. Methylated copies of these genes were quantified in all available stool samples by real-time MSP on a 7900HT fast real-time PCR system (Applied Biosystems). Table 30 shows the results and lists the cut-off (copies) applied. For instance for the most sensitive marker combination panel SFRP2+GATA4+NDRG4+OSMR, cutoff values of the individual markers were SFRP2= 2; GATA4= 4; NDRG4= 0.1 and OSMR= 10. This combination panel had 95% specificity, 87% sensitivity for CRC, and 46% sensitivity for adenomas. The preferred 2-marker combination NDRG4+GATA4 had a 100% specificity, a sensitivity of 73% for CRC, and a 33% sensitivity for adenomas.

Table 30: Performance of marker combinations

	Cutoff (copies) and performance of combination panels *					
	SFRP2+ GATA4+ NDRG4	SFRP2+ NDRG4	SFRP2+ GATA4+ NDRG4+ OSMR (3)	SFRP2+ NDRG4+ OSMR (3)	NDRG4+ OSMR (3)	NDRG4+ GATA4
SFRP 2 cp	1	1	1	1	(-)	(-)
GATA4 cp	4	(-)	4	(-)	(-)	4
NDRG4 cp	0	0	0	0	0	0
OSMR (3)	(-)	(-)	10	10	10	(-)
Sens adenoma	46%	46%	46%	46%	23%	33%
Sens CRC	80%	73%	87%	80%	80%	73%
Specificity	95%	95%	95%	95%	95%	100%

* Marker not used in the combination panel is indicated by (-)

The performance of the most sensitive marker combination panel SFRP2+GATA4+NDRG4+OSMR was evaluated for the different UICC stages. Results are summarized in Table 31.

Table 31: Performance of combination panel SFRP2+GATA4+NDRG4+OSMR for different UICC stages

UICC stage	Neg	Pos	Total
?		1	1
I	1	4	5
II		4	4
III	1	3	4
IV		1	1
Total samples	2	13	15

Example 2

Based on re-expression, 224 different gene assays representing 145 gene promotors were tested on the Base5 methylation profiling platform (data not shown, see reference 2 for details). The 37 most differentially methylated gene sequences assessing 29 gene promoters were validated on retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends (histopathologically normal) using real-time MSP. Several markers reliably detected CRC in those tissue samples (data not shown). The results were confirmed on an independent test set containing 59 samples from patients with cancer other than CRC (20 breast, 21 lung and 22

bladder cancer samples covering stages I-III), 39 non-cancerous controls, 34 carcinomas and 16 adenomas. After testing the non-CRC tissue samples, we had 59 results because 4 were invalid. The individual performance of the 9 best performing tissue markers is shown in Figure 2, when the analytical cut-off was set to give 100% specificity (based on the 39 non-cancerous controls). The most tissue specific markers include: NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT.

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3) EXPERIMENTS ON PLASMA DNA

MATERIALS AND METHODS IN RELATION TO PLASMA DNA

Sample collection and processing

Plasma samples were collected from multiple centers in Germany, The Netherlands and Belgium.
10 ml of blood was obtained per individual using EDTA Vacutainer™ tubes. Individuals with no suspicious findings, adenomas or carcinomas based on colonoscopy were enrolled in the present study. Within 4 hrs from the blood drawing, the plasma fraction was separated from the cell fraction by centrifugation at 1500 g for 15 min (4°C). The plasma was transferred to new tubes and once again centrifuged (1500 g, 15 min, 4°C), after which the supernatant was transferred to new tubes and stored at -80°C until further use. Samples were shipped on dry ice.

Plasma samples from patients with stages I - IV of colorectal cancers and different controls belonging to the following groups were enrolled in this study. Tables 32 and 33 gives an overview of the collected samples sets.

- **Colorectal cancer group:** patients with pathologically confirmed colorectal cancer with stage I to IV (according to the UICC stage grouping)
- **Adenomas**
- **Non-cancer controls:** patients without cancerous disease
- **Cancer controls:** patients with carcinomas other than colorectal cancer

Table 32: Plasma training set 1

Diagnosis group	Sample volume	Number of samples	Notes
Colorectal cancers	**1.2 to 4.5 ml of plasma** (corresponding to 0.07 to 0.27 plasma equivalent of DNA per PCR)	42	StageI - IV Grade 1-3 (81% stage I-III)
Non-cancer controls		34	Symptomatic patients with non-acute conditions
Cancer controls	**4 to 6 ml of plasma** (corresponding to 0.24 to 0.36 plasma equivalent of DNA per PCR)	25	Predominantly ovarian and prostate cancers

Table 33: Plasma training set 2

Diagnosis group	Sample volume	Number of samples	Notes
Colorectal cancers	**1.3 to 4.3 ml of plasma** (corresponding to 0.16 to 0.52 plasma equivalent of DNA per PCR)	78	StageI - IV Grade 1-3 (76% stage I-III)
Adenomas		49	
Non-cancer controls		64	Symptomatic patients with

			non-acute conditions
Cancer controls	**4 to 6 ml of plasma** (corresponding to 0.48 to 0.72 plasma equivalent of DNA per PCR)	25	Predominantly ovarian and prostate cancers

DNA isolation from plasma samples

DNA isolation from plasma samples (1.2 to 6 ml) was performed using an upscaled phenol-chloroform DNA isolation method using the 15 ml of Heavy Phase lock Gel tubes (PLG tubes) (Eppendorf, cat# 0032 005.152) or alternatively the ChargeSwitch® gDNA 1 ml serum kit from Invitrogen (cat# CS11040).

Phenol-Chloroform procedure

Plasma samples were thawed and 1/10 volume of 10x buffer (240 mM EDTA (pH=8.0), 750 mM NaCl), 1/10 volume of 10% SDS and 5 µl of Proteinase K (20 mg/ml stock solution) per 1 ml of sample (e.g. 15 µl for 3 ml of sample) was added to each plasma sample. This mixture was incubated overnight at 48°C at constant shaking (200 RPM).

Subsequently the PLG tube was centrifuged at 2500 RCF for 3 min, sample mixture and approximately the same volume of phenol/chloroform (Invitrogen, cat# 15593049) were added to it. This solution was briefly vortexed, mixed for 10 min using a tube rocker at room temperature and centrifuged for 5 min at 2500 RCF. In case the retrieved sample volume was ≤ 4 ml, an equal volume of phenol/chloroform was added. The upper aqueous layer was phenol/chloroform-treated for a second time.

DNA was precipated from the the upper aqueous layer by adding 5 µl glycogen, 1/10 volume of 7.5 M Ammonium Acetate and 2-2.5 volumes of cold (-20°C) 100% ethanol. Tubes were gently inverted and incubated at -20°C for at least 1 h, followed by a centrifugation step at 17000 RCF for 30 min (4°C). Ethanol was carefully removed by pipetting. Pellets were washed with 2 ml freshly prepared 70% ethanol, vortexed gently and submitted to a centrifugation step at 17000 RCF for 15 min at 4°C. After careful removal of the remaining ethanol, pellets were air dried and resuspended in 45 µl of LoTE pH 8.0. The isolated DNA is stored at -80°C until further processing. This method allowed an average DNA recovery of ≅ 120 ng per ml of plasma.

ChargeSwitch® gDNA 1 ml serum kit

Plasma samples are thawed and DNA is isolated using the ChargeSwitch® gDNA 1 ml serum kit according to the manufacturer's instructions with the exception that the procedure is upscaled for larger sample volumes using the MagnaBot® large volume magnetic separation device from Promega (Cat# V3471). Results are presented in Table 41.

DNA modification

The complete content of DNA isolated in above procedure was subjected to sodium bisulfite treatment (BT) using the EZ-96 DNA Methylation kit from Zymo Research (Cat# D5003) performed on a pipetting robot (Tecan Freedom EVOII, Roma, Liha, Mca, Te-Vacs). Briefly, 45 µl of plasma DNA sample was mixed with 5 µl of M-Dilution Buffer (provided in kit) and incubated at 37°C for 15 min shaking at 1100 RPM. This mixture was further incubated with 100 µl of diluted CT

conversion reagent (provided in kit) shaking at 70°C for 3 hours (protected from light). Subsequently the modified DNA was desalted and desulfonated according to manufacturer's instructions and eluted in either 40 µl or 20 µl of Tris-HCl 1mM pH8.0, depending on the applied concentration procedure. The eluted material was stored at - 80°C until further processing.

DNA amplification

Real-time MSP was performed on a 7900HT fast real-time PCR cycler from Applied Biosystems.

2.4 µl of the modified DNA was added to a PCR mix (total volume 12 µl) containing home-made buffer solution (final concentrations are summarized: 16.6mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM ß-mercaptoethanol), dNTPs (5 mM; Amersham Biosciences cat# 27-2035-02), methylation specific forward primer (6 ng), methylation specific reverse primer (18 ng), molecular beacon (0.16 µM) and Jumpstart DNA Taq polymerase (0.4 units; Sigma Cat# D9307).

Cycling conditions are specified in Table 34.

A standard curve was included (9.6×10^5 - 9.6 copies) to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

Table 34: Cycling profile

1	Activation	95°C	5 min
2	Denaturation	95°C	30 sec
3	Annealing and data	57°C (51°C for APC)	30 sec

	collection		
4	extension	72°C	30 sec
5	cycling	Repeat step 2 to 4, 45 times	

RESULTS

Marker identification and validation in tissue and plasma samples.

Assay validity rate in tissue and plasma:

293 FFPE and 317 plasma samples were processed using real-time MSP (Table 35). The real-time MSP assays produced valid results in 98% of the FFPE samples and in 100% of the plasma samples.

Table 35: Summary of samples evaluated by real-time MSP

Sample Sets	Sample Types	Sample Numbers	Valid Tests [%]
Tissue Training Set	Cancer	65	65/65 [100]
	Controls	76	74/76 [97]
	Total	141	139/141 [99]
Tissue Test Set	CRC	34	34/34 [100]
	Controls	39	39/39 [100]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	152	148/152 [97]
Tissue Sets combined	CRC	99	99/99 [100]
	Controls	115	113/115 [98]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	**293**	**287/293 [98]**
Plasma Training set (1)	Cancer	42	42/42 [100]
	Controls	34	34/34 [100]
	Other cancers	25	25/25 [100]
	Total	101	101/101 [100]

Plasma Training set (2), increased plasma equivalent of DNA per real-time MSP assay	Cancer Adenoma Controls Other cancers Total	78 49 64 25 216	78/78 [100] 49/49 [100] 64/64 [100] 25/25 [100] 216/216 [100]
Plasma Sets combined	Cancer Adenoma Controls Other cancers **Total**	120 49 98 50 **317**	120/120 [100] 49/49 [100] 98/98 [100] 50/50 [100] **317/317 [100]**

Marker identification

Using re-expression profiles of colon cancerous cell lines, candidate genes were identified and the most promising markers (224 different gene assays representing 145 gene promoters) were tested on tissue using the Base5 methylation profiling platform (data not shown, see Straub, J. et al for details). Promoter sequences were linked with gene expression to identify epigenetically silenced genes. An established pharmacologic unmasking strategy (5-aza-2'-deoxycytidine (DAC) and trichostatin A (TSA)) for re-expression analysis of epigenetically targeted genes was combined with proprietary advanced bioinformatics tools to identify genes prone to promoter methylation.

Marker selection in colon tissue

Marker candidates identified by re-expression were screened using 37 real-time methylation specific PCR (real-time MSP) assays. These assays were used to assess the methylation status of 29 gene promoters in 293 formalin-fixed paraffin-embedded (FFPE) tissue samples collected from various clinics. Samples included 99 carcinomas of various stages,

16 adenomas, 63 samples from patients with cancer other than CRC (20 breast [stages I-III], 22 bladder [stages I-III], 21 lung [stages I and II]), 39 samples from patients with no evidence of cancer and 76 distant resection ends (histopathologically normal) from CRC patients. These samples were divided into training and independent test sets, and used to select the gene methylation assays best able to discriminate between cancerous and non-cancerous samples. The training set included retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends. Using the 10 best performing genes the results were confirmed on an independent test set containing 59 samples from patients with cancer other than CRC, 39 non-cancerous controls and 50 cancer cases (34 carcinomas and 16 adenomas). The individual performance of the 10 best performing tissue markers OSMR, SFRP1, GATA4, SFRP2, NDRG4, ADAM23, GATA5, MGMT, APC and JPH3 is shown in FIG.7, when the analytical cut-off was set to give 100% specificity, except for JPH3 where a specificity of 95% was obtained (based on the 39 non-cancerous controls). Corresponding primer and beacon sequences are summarized in Table 3 (above). In addition to the colon test genes, the independent reference gene ß-Actin (ACT) was also measured. The ratios between the colon test genes and ACT were calculated, and are the test result of the assay. The samples were classified as methylated, non-methylated, or invalid based on the decision tree shown in Figure 5.

Complementarity of markers

The different markers were tested on their complementarity. Several marker combinations reliably detected CRC with high specificity and sensitivity. Results of the best 2 marker

combinations are summarized in Table 36. For 100% specificity, sensitivities ranged between 94 to 100%.

Table 36: Performance of 2 combinations of the markers reliably detecting CRC and adenomas when using real-time MSP (tissue test set: 34 carcinomas, 16 adenomas, 39 controls)

	Panel 1 (OSMR, GATA4, ADAM23)		
Samples	# detected / tested	Sensitivity % [95% CI]	Specificity % (# detected / tested)
34 carcinomas 39 controls	33/34	97 [91 - 100]	100 (0/39)
16 adenomas 39 controls	16/16	100	100 (0/39)
50 neoplasms (34 carcinomas and 16 adenomas) 39 controls	49/50	98 [94 - 100]	100 (0/39)
	Panel 2 (OSMR, GATA4, GATA5)		
Samples	# detected / tested	Sensitivity % [95% CI]	Specificity % (# detected / tested)
34 carcinomas 39 controls	32/34	94 [86 - 100]	100 (0/39)
16 adenomas 39 controls	16/16	100	100 (0/39)
50 neoplasms (34 carcinomas and 16 adenomas) 39 controls	48/50	96 [90 - 100]	100 (0/39)

Marker testing in plasma

Eight of the best performing markers in tissue were assessed (OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC) on 101 available plasma samples from multiple centers (plasma

training set 1: Table 32). These plasma samples included 34 samples with no suspicious findings, 25 samples from patients with cancers other than colon cancer and 42 samples from patients covering all stages of CRC, with 81% representing stages I-III of disease.

DNA was isolated following the upscaled phenol-chloroform procedure; subsequently the whole DNA sample was modified as described above. The plasma training set 1 was eluted in 40 µl of BT elution volume of which 2.4 µl was subjected to real-time MSP, the 2.4 µl of eluted DNA corresponds to an equivalent of 0.07 to 0.36 ml of original plasma sample which went into the isolation procedure (= 0.07 to 0.36 plasma equivalent of DNA per PCR).

The individual performance (% sensitivity) of the 8 gene assays in plasma samples is shown in FIG.8, sensitivity values ranging from 14 to 33%. Corresponding specificity values are displayed in Table 37. Obtained specificity values ranged from 97 to 100%.

Five of the best performing markers in training set 1 were further studied with an additional, independent sample set prospectively collected from multiple centers (plasma training set 2: Table 33). Reducing the number of gene assays from 8 to 5 resulted in fewer assays per sample and a greater aliquot of plasma equivalent of DNA was added per PCR reaction. The modified DNA from sample set 2 was more concentrated by eluting in 20 µl instead of 40 µl of BT elution volume. 2.4 µl eluted DNA from sample set 2 was further processed through real-time MSP, this corresponds to

0.16 to 0.72 ml plasma equivalent of DNA per PCR depending on the plasma volume prior to DNA isolation.

The plasma samples of training set 2 included 64 samples with no suspicious findings, 49 adenomas, 25 samples from patients with cancers other than colon cancer and 78 samples from patients covering all stages of CRC, with 76% representing stages I-III of disease. The individual performance (% sensitivity) of the 5 gene assays is shown in FIG. 8 with corresponding specificity values displayed in Table 37. Specificity values ranged from 96 to 99%, with sensitivity ranging from 23 to 47%.

Four candidate methylation markers were found to result in the best sensitivity and specificity in plasma samples: OSMR, NDRG4, GATA5 and ADAM23; performance of this plasma panel is shown in Table 38. Performance characteristics (stages I-III CRC) of this panel of 4 methylation genes demonstrated 73% sensitivity and 92% specificity when optimized for sensitivity, whereas 64% sensitivity and 98% specificity was obtained when optimizing for specificity. Sensitivity can be further improved (from 64% to 68%) when samples with a plasma volume less than 2 ml prior to DNA isolation are excluded from analysis. Results are presented in Table 39.

Table 37: Individual gene assay performance displaying % specificity for both plasma training sets and % sensitivity for adenomas in plasma training set 2

	OSMR	SFRP1	NDRG4	GATA5	ADAM23	JPH3	SFRP2	APC
% Specificity (all 59 controls), plasma set 1	100	98	100	97	98	97	97	97
% Specificity (all 89 controls), plasma set 2: increased plasma equivalent of DNA per real-time MSP assay	99	96	99	99	97	N/A	N/A	N/A
% Sensitivity adenomas plasma set 2: increased plasma equivalent of DNA per real-time MSP assay	2	2	0	6	2	N/A	N/A	N/A

Table 38: Performance of a plasma marker panel using real-time MSP (independent of recovered plasma volume prior to DNA isolation)

		Plasma panel (optimized for sensitivity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 1	Stages I-III CRC All Stages CRC All Controls	50 (17/34) 60 (25/42) [45 - 83]	 97 (2/59) [93 - 100]
Plasma training set 2 (increased plasma equivalent of DNA per real-time MSP assay)	Stages I-III CRC All Stages CRC Adenomas All Controls	73 (43/59) 73 (57/78) [63 - 83] 12 (6/49)	 92 (7/89) [86 - 98]
		Plasma panel (optimized for specificity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 1	Stages I-III CRC All Stages CRC All Controls	N/A	N/A
Plasma training set 2 (increased plasma equivalent of	Stages I-III CRC All Stages CRC Adenomas All Controls	64 (38/59) 64 (50/78) [53 - 75] 6 (3/49)	 98 (2/89) [95 - 100]

DNA per real-time MSP assay)			

Table 39: Performance of a plasma marker panel using real-time MSP using at least 2 ml of plasma prior to DNA isolation

		Plasma panel (optimized for sensitivity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 2 (increased plasma equivalent of DNA per real-time MSP assay)	Stages I-III CRC All Stages CRC Adenomas All Controls	73 (41/56) 74 (54/73) [64 - 84] 12 (6/49)	 92 (7/89) [86 - 98]
		Plasma panel (optimized for specificity) OSMR, NDRG4, GATA5 and ADAM23	
Sample sets	Sample groups	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Plasma training set 2 (increased plasma equivalent of DNA per real-time MSP assay)	Stages I-III CRC All Stages CRC Adenomas All Controls	68 (38/56) 67 (49/73) [56 - 76] 6 (3/49)	 98 (2/89) [95 - 100]

Average DNA recovery yield from plasma samples

Plasma DNA (collected after double centrifugation step) from colorectal cancer patients was isolated according to the phenol/chloroform procedure and quantified using the PicoGreen dsDNA quantitation kit from Molecular Probes. The average plasma DNA recovery yield was 117 ng/ml of plasma, with a range of 41 to 384 ng/ml (data obtained from 25 patients).

Table 40: Average DNA recovery yield plasma samples

Sample	ng/ml plasma
1	41
2	66
3	264
4	163
5	54
6	121
7	87
8	107
9	53
10	121
11	88
12	201
13	53
14	47
15	384
16	87
17	115
18	107
19	70
20	72
21	122
22	146
23	71
24	195
25	94

Phenol/Chloroform procedure versus ChargeSwitch® using plasma samples

This experiment was carried out to show the isolation of DNA from plasma by using the method of this invention. Plasma volumes ranging from 2.5 to 6 ml were processed according to the above discussed upscaled phenol/chloroform and ChargeSwitch® isolation procedure. Plasma derived from ovarian, prostate and colon blood samples were investigated. The objective was to isolate DNA (according to both methods) and further process the samples in parallel through bisuphite treatment and β-Actin real-time MSP to address the sample quality and DNA yield. The corresponding β-Actin copies for both isolation procedures are summarized in Table 16.

Table 41: β-Actin copies phenol/chloroform versus ChargeSwitch® isolation procedure

Sample number	Sample origin	Plasma volume (ml)	B-Actin copies Phenol	B-Actin copies ChargeSwitch
1	ovarian cancer	6.0	4349	863
2	ovarian cancer	6.0	2710	466
3	ovarian cancer	6.0	3922	967
4	ovarian cancer	6.0	758	490

5	ovarian cancer	6.0	4201	423
6	ovarian cancer	6.0	2644	139
7	ovarian cancer	6.0	1472	187
8	prostate cancer	2.6	145	7
9	colon cancer	2.5	317	52
10	pos control cell line	N/A	8702	1314

Updated results for plasma training set 2.

Corrected information was received from the clinics about plasma training set 2. For plasma training set 2: the cancer cases remained the same, a new category of "unknown" was created, the number of controls was 52 (instead of former 64) and the adenoma cases were 39 (instead of former 49). This allowed re-classification of sample types as provided in Table 42. Since the corrected information classified a number of unknown cancer cases (controls) as early stage cancers, additional conclusions on detection of early stage cancers could be drawn. As shown in table 43, the plasma panel allowed very sensitive detection (70%) of early stage samples. Improved detection could be obtained by excluding samples with a plasma volume less than 2 ml (Table 44)

Table 42: Summary of samples tested by real-time MSP and evaluablility rate

Sample sets	Sample types	Sample numbers	Valid tests [%]
Tissue training set	CRC	65	65/65 [100]
	Controls	76	74/76 [97]
	Total	141	139/141 [99]
Tissue test set	CRC	34	34/34 [100]
	Controls	39	39/39 [100]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	152	148/152 [97]
Tissue sets combined	CRC	99	99/99 [100]
	Controls	115	113/115 [98]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	**293**	**287/293 [98]**
Plasma training set (1)	CRC	42	42/42 [100]
	Controls	34	34/34 [100]
	Other cancers	25	25/25 [100]
	Total	101	101/101 [100]
Plasma training set (2)	CRC	78	78/78 [100]
	Adenoma	[redacted]	49/49 [100]
	Controls	[redacted]	64/64 [100]
	Other cancers	25	25/25 [100]
	[redacted]	[redacted]	22/22 [100]
	Total	216	216/216 [100]
Plasma sets combined	CRC	120	120/120 [100]
	Adenoma	39	49/49 [100]
	Controls	86	98/98 [100]
	Other cancers	50	50/50 [100]
	Unknown	22	22/22 [100]
	Total	**317**	**317/317 [100]**

Sample groups (plasma training set 2)	Plasma panel OSMR, GATA5, NDRG4 and ADAM23			
	optimized for sensitivity		optimized for specificity	
	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Early stages CRC (0-II)	70% (23/33) [54-86]	92% (6/77) [86-98]	58% (19/33) [41-75]	99% (1/77) [96-100]
All stages CRC	73% (57/78) [63-83]		64% (50/78) [53-75]	
Adenomas	10% (4/39)		5% (2/39)	
Controls				

Table 43: Performance characteristics of a 4-gene marker panel using plasma set 2

Sample groups (plasma training set 2)	Plasma panel OSMR, GATA5, NDRG4 and ADAM23			
	optimized for sensitivity		optimized for specificity	
	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Early stages CRC (0-II)	70% (23/33) [54-86]	92% (6/77) [86-98]	58% (19/33) [41-75]	99% (1/77) [96-100]
All stages CRC	74% (54/73) [64-84]		67% (49/73) [56-78]	
Adenomas	10% (4/39)		5% (2/39)	
Controls				

Table 44: Performance characteristics of a 4-gene marker panel using plasma set 2

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4) N-Myc downstream regulated gene 4 (NDRG4) promoter methylation is a sensitive and specific biomarker for colorectal cancer

Abstract

Background and aims: N-Myc downstream regulated gene 4 (NDRG4), a gene involved in cellular differentiation and neurite formation, is one of the four members of the NDRG family. Here we address the role of NDRG4 promoter methylation in CRC (CRC).

Methods: NDRG4 promoter methylation was analyzed in CRC cell lines, well characterised series of normal colon mucosa, colorectal adenomas, carcinomas and other neoplasias using methylation specific PCR (MSP) and bisulfite sequencing. NDRG4 promoter methylation was also analyzed in fecal DNA of CRC patients and controls using quantitative MSP. Loss of heterozygosity (LOH) mapping of the NDRG4 locus and mutation analysis using direct sequencing of NDRG4 coding exons and

their flanking intronic regions were performed. NDRG4 mRNA and protein expression was studied using RT-PCR and immunohistochemistry respectively.

Results: NDRG4 promoter methylation is observed in 7/8 CRC cell lines. The prevalence of NDRG4 promoter methylation in CRC tissue is 86% (71/83) compared to 4% (2/48) in normal colon mucosa. A second, independent series of CRCs confirmed the high prevalence (69%, 127/183) of NDRG4 methylation. NDRG4 methylation was also observed in 81% (13/16) of oesophageal adenocarcinomas and 77% (17/22) of gastric cancers while no or little methylation was observed in skin (0/8), kidney (1/10), ovary (0/20), prostate (0/10), breast (0/16) and oesophageal squamous cell cancers (0/12). NDRG4 promoter methylation can be detected in fecal DNA of 76% (16/21) of CRC patients, while only 3% (2/67) of control patients tested positive yielding a sensitivity of 76% and a specificity of 97%. No mutations were found and 30,5% of tumors showed LOH on the NDRG4 locus. Expression of NDRG4 is decreased at the RNA and protein level in CRC when compared to normal tissue.

Conclusions: NDRG4 is frequently methylated in CRC cell lines, colorectal adenomas and carcinomas and other adenocarcinomas of the gastrointestinal tract. NDRG4 promoter methylation in fecal DNA can be used as a sensitive and specific biomarker for the detection of CRC.

Introduction

Previous microarray experiments to identify genes which are epigenetically regulated in tumor endothelial cells revealed 81 genes that are downregulated in tumor endothelial cells

and reexpressed after 5-aza-2'-deoxycytidine (DAC) and trichostatin A (TSA) treatment. Silencing of these genes in tumor- endothelial cells was associated with promoter histone H3 deacetylation and loss of H3 lysine 4 methylation, however did not involve DNA methylation of promoter CpG islands. Interestingly, 21 of these 81 genes (26%) have been reported to be hypermethylated and silenced in various tumor types suggesting that many of the identified gene promoters have the potential to be regulated by promoter methylation in tumor cells (Hellebrekers, Melotte et al. 2007). Amongst the identified CpG island containing genes is N-myc downregulated gene-4 (NDRG4), also known as Smap-8 and Bdm1. NDRG4 is part of the NDRG family which consists of four members, NDRG1, -2, -3 and -4 which have an amino acid sequence homology of 57-65% (Zhou, Kokame et al. 2001; Qu, Zhai et al. 2002). Phylogenetic analysis verified two subfamilies, one consisting of NDRG1 and -3 and the other consisting of NDRG-2 and -4 (Qu, Zhai et al. 2002). NDRG1 is the most extensively studied member of the NDRG family. Expression of NDRG1 is often downregulated in cancer cells (van Belzen, Dinjens et al. 1997; Kurdistani, Arizti et al. 1998; Guan, Ford et al. 2000; Bandyopadhyay, Pai et al. 2003; Bandyopadhyay, Pai et al. 2004; Shah, Kemeny et al. 2005) and upregulated by DAC treatment (Guan, Ford et al. 2000; Bandyopadhyay, Pai et al. 2004). In addition, NDRG2 has also been described as candidate tumor suppressor gene (Deng, Yao et al. 2003; Lusis, Watson et al. 2005) and reported to be methylated in meningiomas (Lusis, Watson et al. 2005) and different cancer cell lines (Liu, Wang et al. 2007). So far, the function of NDRG3 and NDRG4 in cancer has not been addressed. The NDRG4 gene is located on chromosome 16q21-q22.3, spans 26kb and contains 17 exons

covering the entire sequence of three cDNA isoforms NDRG4-B, NDRG4-Bvar and NDRG4-H. NDRG4 mRNA is predominantly present in the cytoplasm. At present, expression of NDRG4 has only been described in brain and heart using Northern blot analysis. The molecular characterization of NDRG4 and the role of this protein in the nervous system has mainly been investigated in the rat (Nakada, Hongo et al. 2002; Ohki, Hongo et al. 2002; Maeda, Hongo et al. 2004; Hongo, Watanabe et al. 2006). NDRG4 protein may participate in processes that lead to cellular differentiation and neurite formation (Ohki, Hongo et al. 2002).

Here, we report NDRG4 to be expressed in normal colon mucosa and downregulated in colon cancer tissue. In addition, NDRG4 promoter methylation, loss of heterozygosity (LOH) and mutational inactivation were examined. We identified the NDRG4 promoter as being frequently methylated in CRC and other neoplasias of the gastrointestinal tract and investigated its potential as a biomarker in stool of CRC patients and controls.

Materials and Methods

Cell lines, study population and tissues

CRC cell lines HT29, SW480, Caco2, Colo205, RKO, LS174T, HCT116 and SW480 were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Hyclone). To investigate reexpression of NDRG4 following inhibition of DNA methyltransferases, HCT116 and RKO were treated with 1 µM DAC (Sigma).

NDRG4 promoter methylation was investigated in well-characterized series of colorectal carcinomas, adenomas and

controls (FIG.9). The first series consists of formalin-fixed, paraffin-embedded CRCs (n=90) of patients over 50 years of age which were retrospectively collected from the archive of the dept. of Pathology of the University Hospital Maastricht. When present, also normal (n=79) and adenoma (n=60) tissue was collected from these patients. Histologically normal biopsy material from patients undergoing endoscopy for non-specific abdominal complaints (n=51), adenoma biopsies (n=22) from patients who did not develop CRC within 10 years, and resected colon mucosa of patients with various inflammatory bowel conditions (n=33) were selected as control tissue. This last group includes Crohn's disease (n=1), colitis ulcerosa (n=6), non-specific inflammation (n=9) and diverticulitis (n=18). A second independent series of CRCs (n=200) was randomly selected from the prospective Netherlands Cohort Study on diet and cancer (NLCS), which has been described in detail elsewhere (van den Brandt, Goldbohm et al. 1990; Brink, de Goeij et al. 2003). Series characteristics are shown in supplemental table 1 In addition, archival, formalin-fixed, paraffin-embedded skin- (n = 8), kidney- (n = 10), ovary- (n = 10), prostate- (n = 10), breast- (n = 15), stomach- (n = 22) and oesophagus (n= 28) cancer tissue was analyzed for NDRG4 promoter methylation. This study was approved by the Medical Ethical Committee (MEC) of the Maastricht University and the University Hospital Maastricht.

Table 45: Series characteristics

	Age*	Sex†	Location‡	
			Proximal	Distal
CRC+				
Normal tissue	71.0 ± 8.6	41/38	40/75 (53%)	35/75 (47%)
Adenoma tissue	71.7 ± 7.9	32/30	26/59 (44%)	33/59 (56%)
Carcinoma tissue	71.5 ± 8.3	44/46	49/88 (56%)	39/88 (44%)

CRC-				
Normal tissue	65.2 ± 9.0	22/29	13/39 (33%)	26/39 (67%)
Adenoma tissue	63.1 ± 7.6	16/6	6/18 (33%)	12/18 (67%)
Inflamed tissue	65.3 ± 10.1	14/19	10/26 (39%)	16/26 (62%)
P-value	*<0.001*	*NS*		*NS*

	Carcinoma tissue		**CRC+** Adenoma tissue	**CRC-** Adenoma tissue
Histological type		Histological type		
Adenocarcinoma	72/90 (80%)	Tubular	39/62 (63%)	16/22 (73%)
Mucinous carcinoma	18/90 (20%)	Tubulovillous	22/62 (36%)	6/22 (27%)
		Villous	1/62 (2%)	0/22 (0%)
Differentiation:		Dysplasia		
Poor	8/90 (9%)	Lowgrade	54/62 (87%)	22/22 (100%)
Moderate	70/90 (78%)	Highgrade	8/62 (13%)	0/22 (0%)
Well	12/90 (13%)			
TNM stage:				
I	13/90 (14%)			
II	29/90 (32%)			
III	36/90 (40%)			
IV	12/90 (13%)			

Table 4.5: Patient characteristics NDRG4b
**years ± SD, analyzed by One-way ANOVA*
†Male/Female, analyzed by Pearson's χ^2
‡analyzed by Pearson's χ^2. Location could not be traced for all samples explaining different total sample numbers

CRC+: colorectal cancer patients
CRC-: patients without colorectal cancer
NS: not significant
TNM stage: 'Tumour Node Metastasis' Staging

DNA-isolation from tissues and cell lines

A 5 µm section of each tissue block was stained with haematoxylin and eosin and revised by a pathologist (AdB). Five sections of 20 µm were deparaffinated prior to DNA-isolation. DNA was extracted from these tissue samples and from cell lines using the Puregene® DNA isolation kit (Gentra systems) according to the manufacturers instructions. In brief, cell lysis solution and proteinase K (20 mg/ml, Qiagen) were added to the tissue samples and incubated overnight at 55°C. Subsequently, DNA was extracted

for 72 h at 37°C, protein was removed, and DNA was precipitated using 100% 2-propanol. Finally, DNA was rehydrated in hydration buffer.

Collection and preparation of fecal DNA

Colonoscopy negative control stool samples (n=67) were obtained from a population of healthy subjects over 50 years of age which are being screened within the framework of a workplace-based community CRC screening study at the University Hospital Maastricht. The Medical Ethical Committee (MEC) of the Maastricht University, the University Hospital Maastricht and the Dutch 'Wet op Bevolkingsonderzoek' (WBO) is approving this screening study. Stool samples from colonoscopy confirmed CRC patients (n=21) covering all CRC stages were collected at the Free University Medical Center in Amsterdam. For recovery of human DNA, whole stool samples were homogenized in a 7 excess volume of stool homogenization buffer (Exact sciences, Marlborough, MA, USA) and aliquoted in portions of 32 ml containing the equivalent of 4g of stool each. Single aliquots were centrifuged and the supernatants were incubated with 80 units per ml RNase A for 60 minutes at 37°C. Total DNA was then precipitated using sodium acetate isopropanol (PH 5.2), washed with 70% ethanol and resuspended in 4ml 1xTE (pH 7.4). 400 µl 10x buffer (240mM EDTA (pH 8.0), 750 mM NaC), 400 µl 10% SDS and 20 µl Proteinase K (20 mg/ml) was added, samples were incubated overnight at 48°C at constant shaking and centrifuged the next day. Additionally, 5 ml of phenol-chloroform-isoamylalcohol was added and samples were incubated for 10 minutes at RT before centrifugation. The phenol- chloroform-isoamylalcohol extraction was repeated, the aqueous layer

was subsequently transferred in a new tube, DNA was precipitated, washed and pellets were resuspended in 2 ml of LoTE (pH 8.0).

Sodium bisulfite conversion, methylation-specific PCR and sodium bisulfite sequencing

Sodium bisulfite modification of 500 ng genomic DNA was performed using the EZ DNA methylation kit (ZYMO research Co., Orange, CA) according to the manufacturer's instructions. NDRG4 MSP analysis on bisulfite treated DNA retrieved from cell lines and formalin-fixed, paraffin embedded tissue was facilitated by first amplifying the DNA with flanking PCR primers which amplify bisulfite-modified DNA but do not discriminate between methylated or unmethylated DNA. This PCR product was used as a template for the MSP reaction (Herman, Graff et al. 1996; van Engeland, Weijenberg et al. 2003). Flank primers, MSP primers and PCR conditions are listed in table 2**(see above)**. All PCRs were performed with controls for unmethylated DNA (DNA from normal lymphocytes), methylated DNA (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs), and a control without DNA. Ten µl of each MSP reaction were directly loaded onto 2% agarose gel and visualized under UV illumination. For sequencing of sodium bisulfite-converted DNA, PCR products were amplified and cloned using the TOPO-TA cloning kit (Invitrogen, Breda, the Netherlands). Single colonies were picked and sequenced using an automated sequencer (Applied Biosystems, Foster City, CA). Primer sequences used are SEQ ID NO: 570 5'-GATYGGGGTGTTTTTTAGGTTT-3' (sense primer) and SEQ ID NO: 6 5'- CRAACAACCAAAAACCCCTC-3' (antisense primer).

Quantitative MSP

Quantitative real-time MSP was performed using a 7900HT real-time PCR system (Applied Biosystems). 2.4 µl of the modified DNA (equivalent to 2,5 µg unconverted DNA) was added to a PCR mix (total volume 12 µl) containing buffer (16.6mM (NH4)2SO4, 67 mM Tris (pH 8.8), 6.7 mM MgCl2, 10 mM ß-mercaptoethanol), dNTPs (5 mM), forward primer (6 ng), reverse primer (18 ng), molecular beacon (0.16 µM), BSA (0.1 µg), and Jumpstart DNA Taq polymerase (0.4 units; Sigma Aldrich). The PCR program was as follows: 5 minutes 95°C, followed by 45 cycles of 30 seconds 95°C, 30 seconds 57°C, and 30 seconds 72°C, followed by 5 minutes 72°C. Primer sequences used are SEQ ID NO: 17 5' - GTATTTTAGTCGCGTAGAAGGC - 3' (forward primer), SEQ ID NO: 18 5' - AATTTAACGAATATAAACGCTCGAC - 3' (reverse primer) and SEQ ID NO: 19 5'-FAM-CGACATGCCCGAACGAACCGCGATCCCTGCATGTCG-3'-DABCYL (molecular beacon). A standard curve ($2x10^{6-20}$ copies) was included to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve.

Loss of Heterozygosity Analysis

Allelic status was analyzed by PCR amplification with specific primer pairs flanking polymorphic microsatellite loci. The fluorescent dye-labeled microsatellite markers DS16S3089 (forward primer: SEQ ID NO: 526 AGCCCTGCCTGATGAA; reverse primer: SEQ ID NO: 527 TGTGTGGGTAGCACCAA) and DS16S3071 (forward primer: SEQ ID NO: 528 AGCTCTCTGATGGGCAGTG; reverse primer: SEQ ID NO: 529 TGGAAGATAGCCCCCAAAT) located on 16q21-22 were selected from genome public database. DS16S3089 is situated 1.9Mb downstream of NDRG4 and DS16S3071 1.8 Mb upstream of NDRG4.

Matched tumor/normal DNA samples were amplified by PCR in a 15µl volume containing 0,25 mM dNTP, 0,3µM primers, 1,5mM MgCl2 and 0,04 units Taq-polymerase (platinum, Invitrogen) using 50ng DNA as template. The reaction mixture was subjected to 3 min of denaturing at 95°C and 30 cycles of 95°C for 1 min, 60°C annealing temperature for 1 min and 72°C for 1 min followed by a final extention step at 72°C for 10 min. PCR products were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA) and analyzed using Genemapper software version 4,0 (Applied Biosystems). Only genotypes demonstrating two different sizes, i.e. heterozygous MS alleles, were used for evaluating allelic status.The allelic ratio was calculated as (N1/N2)/(T1/T2) for the ratio of area values of tumor (T) versus the normal (N) alleles. LOH was defined as an allelic ratio more than 1.35 and less than 0.67.

Mutation analysis

The NDRG4 coding exons and their flanking intronic regions were individually amplified using genomic DNA extracted from paraffine embedded colonic adenocarcinoma tissue. Mutation analysis was examined using the nested PCR approach. The outside PCR was performed with 125 ng genomic DNA, 50 pmol of each forward and reverse primer and 1 units of TaqPolymerase mixture (Invitrogen). DNA amplification was done on a thermal cycler using Thermo-Fast 96-well plates (Corning) starting with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing with an specific temperature for each primer for 30s and extension at 72°C for 30 sec. An additional final extension of 72°C for 5 min was added. Following the outside PCR an inside PCR was done using the same conditions

as the outside PCR. PCR primer sets for each exon, including intron-exon boundary, are provided in detail in supplemental table 3. DNA was purified using the Millipore multiscreen 96 wells plate (Millipore). PCR products were amplified using the BigDye® Terminator v1.1 Cycle sequencing kit and amplified products were sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Table 46: *NDRG4* mutation analysis primer sequences and PCR conditions.

Exon No.	Primer	SEQ ID NO :	Sense primer	SEQ ID NO	Antisense primer	Annealing temperature
2	Outside	530	CCCAGCCCCGACTTGC	531	CTAAGACCTCAAAGGCGCG	56
	Inside	532	TGTCCTTCTCCGCCCGG	531	CTAAGACCTCAAAGGCGCG	62
3	Outside	533	CCCCTCTGTTTGCCTTCC	534	CTGGCCAGGTGGGGTG	56
	Inside	533	CCCCTCTGTTTGCCTTCC	535	GCCAGGTGGGGTGAGGG	62
4	Outside	536	CTGCGTCACCTCATTCCC	537	TCACCGCTCTGGCTGATG	56
	Inside	538	GAGGAGCCAAGAGCGGAGG	537	TCACCGCTCTGGCTGATG	62
5	Outside	539	CCCCTCTGCTCAGCCATAG	540	GCTGGAGACAGGCAGAGGG	56
	Inside	539	CCCCTCTGCTCAGCCATAG	541	GGAGACAGGCAGAGGGGG	56
6	Outside	542	GTAGGTACCCTGAGCCCCC	543	ACCCCTGGGCCCTAGC	56
	Inside	544	CCCTCTGCCTGTCTCCAGC	543	ACCCCTGGGCCCTAGC	62
7	Outside	545	GGAAATGGCACCCCTAGC	547	GGGGGCATGGGGAGAC	56
	Inside	548	GCACCCCTAGCCCTAGAGT	547	GGGGGCATGGGGAGAC	56
8	Outside	549	CCTTGAAGACTTTACAGAGTGTTTC	550	GTATACCCACCCCACCCC	56
	Inside	551	CTGCACCCATCCTGGCC	550	GTATACCCACCCCACCCC	62
9	Outside	552	GGGGTGGGGTGGGTATAC	553	GCTGGGAGGGGCAAATC	56
	Inside	552	GGGGTGGGGTGGGTATAC	554	GGCAAATCCCAGATCACCC	62
10	Outside	555	GCCTCCATCCATCTCCCTG	556	GGCTGCTGATCCCACCC	56
	Inside	557	CATGCCTCCATCCATCTCC	556	GGCTGCTGATCCCACCC	62
11+12	Outside	558	CACCTCTGCCTCTGCCCC	559	CCCCAGTGAGCCCACAGC	56
	Inside	560	CCTCTGCCCCTCCCCC	559	CCCCAGTGAGCCCACAGC	62
13	Outside	561	TGCCTTGGCAATGGGG	562	CAGGGCTGGGGAAGAAAG	56
	Inside	563	CTTGGCAATGGGGGTGG	562	CAGGGCTGGGGAAGAAAG	62
14+15	Outside	564	GGAGCTTGTCCTGGAGTGAG	565	GTGGGGTGGAAATGTACTCAC	56
	Inside	566	TGGAGTGAGGGCCCTGC	565	GTGGGGTGGAAATGTACTCAC	62
16	Outside	567	TGCCCGCCAGTCCTCAG	568	TAAAGGGAACATGAGCCGG	56
	Inside	569	CAGTCCTCAGGCCCATCC	568	TAAAGGGAACATGAGCCGG	56

*Number of cycles in each case was 35

Quantitative reverse transcriptase PCR

Total RNA from cell lines, normal mucosa and tumor tissue was isolated using the Rneasy Mini kit (Qiagen) following the manufacturers instructions. Possible genomic DNA contaminations were removed by DNAse treatment with the RNase-free DNAse set (Qiagen). cDNA synthesis using the

Iscript cDNA synthesis kit (Bio-Rad) was performed. Quantitative real-time (RT-PCR) was performed using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Realtime RT-PCR mixes were composed of 1x iQ SYBR Green Supermix (Bio-Rad), 400 nM of the forward (SEQ ID NO: 3 5'-GGCCTTCTGCATGTAGTGATCCG-3') and reverse (SEQ ID NO: 4 5'-GGTGATCTCCTGCATGTCCTCG-3') primer and cDNA corresponding to 30 ng total RNA per reaction. As standard control, primers targeted against cyclophilin A were used. Reactions were run using the iCycler (Bio-Rad) for 40 cycles at a Tm of 60 °C. The comparative Ct method was used to calculate differences in mRNA expression. To do so, the Ct value of each sample was normalized to the reference gene ([delta]Ct = Ct,sample - Ct,cyclo). Next, the fold difference in expression was calculated as $2^{-[delta][delta]Ct}$, with [delta][delta]Ct = [delta]Ct,samplel - [delta]Ct,control.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin embedded tissue sections (5 µm) of normal colon mucosa and CRC tissue. Sections were deparaffinized in xylene, rehydrated and incubated with 1% methanol for 30 minutes to inactivate the endogenous peroxidase. After blocking, sections were stained with the NDRG4 monoclonal antibody (Abnova Corporation), 1:6000 diluted in Tris-buffered saline (TBS) with 0.1% Tween and 0.5% bovine serum albumin (BSA) and incubated for 60 minutes. Sections were incubated with the secondary antibody poly-HRP-GAM/R/R IgG (Immunologic, Immunovision Technologies) and staining was visualized as a brown precipitate using DAB substrate chromogen (Dako) followed by haematoxylin counterstaining.

Sections incubated without the primary antibody served as a negative control.

Data analysis

We used the Pearson's $\chi 2$ or Fisher's Exact test and the One-way ANOVA, Kruskal-Wallis or Mann-Witney test where appropriate to compare non-parametric and categorical data respectively. Paired samples within the group of cases were analyzed using the McNemar test and the paired T-test to compare non-parametric and categorical data respectively. Logistic regression analysis was used to compare categorical data adjusted for age and location of the tissue since significant differences in age and location of the different tissues were observed between CRC cases and controls. All quoted p-values are two-sided, and a p-value 0.05 or lower was considered statistically significant. All statistical tests were corrected for multiple comparisons using the Bonferroni method. Data analysis was done using SPSS software (version 12.0.1).

Results

NDRG4 promoter methylation and expression in CRC cell lines

The structure of the NDRG4 gene shows a dense CpG island (GC content > 60%, ratio of observed CpG / expected CpG > 0.6 and minimum length 200 bp (Gardiner-Garden and Frommer 1987)) located -556 to +869 relative to the transcription start site as shown in FIG.10. To assay this region for potential methylation we designed two different MSP primer pairs (1 and 2) amplifying overlapping fragments in the CpG island. These primers were initially used to investigate eight CRC cell lines (LS174, HCT116, HT29, RKO, CACO2,

COLO2, SW48 and SW480) for DNA methylation. All cell lines except SW480 were methylated as analyzed by MSP using both primer pairs as shown in FIG.11a. To further investigate the pattern of CpG island methylation we performed sodium bisulfite sequencing of HCT116 and SW480. The promoter region spanning 39 CpG sites was PCR-amplified using sodium bisulfite-modified genomic DNA as template and six clones of each cell lines were sequenced. Bisulfite sequencing confirmed MSP data in that HCT116 showed almost complete methylation at 39 sites as depicted in FIG.11b, whereas SW480 showed almost no methylated CpG sites. Endogenous NDRG4 mRNA levels in CRC cell lines HCT116 and RKO were significant increased after treatment with DAC (FIG.11c).

Methylation of NDRG4 in normal and CRC tissue

Methylation of NDRG4 was confirmed in three pairs of primary tumors and matched normal colonic mucosa by sodium bisulfite sequencing. The results depicted in FIG.12a show dense methylation of the three tumor samples while almost no methylation was observed in the normal colon mucosa. Interestingly, the density of methylation was higher in the upstream region of the NDRG4 CpG island when compared to more downsteam region as shown in FIG.12a.

Subsequently, the methylation status of NDRG4 was investigated in colorectal carcinoma, adenoma and normal colorectal mucosa using two different primer pairs (1 and 2). The methylation frequenties using both primer pairs are depicted in table 47. A significant difference (table 47, p=0.042 10-7) was observed in methylation frequencies in normal mucosa of the control group (2/48 (4%)) compared to cancer tissue of CRC patients (71/83 (86%)) using primer pair 2. In addition, we compared NDRG4 promoter methylation

in adjacent normal mucosa tissue of CRC patients (9/78 (12%)) and the normal mucosa of non-cancerous patients (2/48 (4%) but did not find a significant difference among these two groups (table 47). Furthermore, to investigate NDRG4 methylation in premalignant lesions, we compared adenomas obtained from CRC patients that developed synchronously or metachronously to the tumour and adenomas obtained from patients that did not develop CRC after 10 years of follow-up. We observed a higher prevalence of NDRG4 methylation in adenomas from CRC patients although these differences did not reach statistical significance (table 47).

Table 47: Methylation frequencies (%) of normal, adenoma, carcinoma tissue from CRC patients and normal, adenoma tissue of non-cancerous patients. Methylation differences are analyzed by logistic regression adjusted for age (NDRG4p1, p2) and location (NDRG4 p1)

	Carcinoma tissue	Controls normal	*P*	Normal tissue controls	Normal tissue CRC+	*P*	Adenoma tissue Controls	Adenoma tissue CRC+	*P*
NDRG4 p1	71%	0%	0.02×10^{-2}	0%	3%	NS	13%	41%	NS
NDRG4 p2	86%	4%	0.042×10^{-7}	4%	12%	NS	55%	66%	NS

Abbreviations: CRC+, colorectal cancer patients; P, P-value; NS, not significant

To confirm the high prevalence of NDRG4 promoter methylation in CRC, we analyzed a second independent series of 183 CRC samples. Comparable to the results of the first study series we observed that 70% (127/183) of CRC patients presented NDRG4 methylation.

Further analysis of the clinicopathologic features of patients with primary CRC with regard to NDRG4 promoter methylation did not reveal any association with age at

diagnosis, sex, location of the tumor or the TNM stage for both independent series using primer 2 (table 49).

To investigate NDRG4 promoter methylation during cancer progression we compared the frequency of methylation from normal mucosa to adenoma and carcinoma tissues in patients for which all the three tissues were available (table 48). Our results show that NDRG4 is significantly (table 48, p<0.02 10-2) more frequently methylated in carcinomas (84%) compared to normal mucosa adjacent to the tumor (16%). In addition to the carcinomas, adenoma samples from CRC patients also exhibit significantly (table 48, p<0.03 10-3) higher NDRG4 methylation frequencies (61%) compared to normal colon samples (14%). Finally, NDRG4 methylation was increased in carcinoma tissues (81%) compared to adenoma samples (63%) although this enhancement was not significant (primer pair 2, table 48).

Table 48: *NDRG4* Methylation frequencies (%) of carcinoma tissue, adenoma and normal tissue from colorectal cancer patients. Methylation differences were analyzed by Mc Nemar test.

CRC patients	Normal tissue	Adenoma tissue	*P*	Normal tissue	Carcinoma tissue	*P*	Adenoma tissue	Carcinoma tissue	*P*
NDRG4 p1	0%	34%	0.003	0%	73%	$0.01x10^{-4}$	39%	76%	0.012
NDRG4 p2	14%	61%	$<0.03x10^{-3}$	16%	84%	$<0.02x10^{-2}$	63%	81%	NS

Frequencies may vary because of missing data for some variables.
Abbreviations: CRC+, colorectal cancer patients; P, P-value; NS, not significant

The different series were analyzed using two different primer pairs 1 and 2 amplifying overlapping fragments in the CpG island, as depicted in FIG.10. Using primer pair 1 we observed overall the same results compared to primer pair 2

however we found an increase of NDRG4 methylation for all the subgroups using primer pair 2 compared to primer pair 1. Interestingly, we found a significant difference (table 2, p=0.012) in promoter methylation in adenomas of CRC patients (55/77 (41%)) compared to the carcinomas (55/77 (71%)) which was not observed using primer pair 2. In addition, comparing the NDRG4 methylation status of adenomas obtained from CRC patients that developed synchronously or metachronously to the tumour (24/58(41%)) and adenomas obtained from patients that did not develop CRC (4/31 (13%) we observed a enormous increase of NDRG4 methylation in adenomas from CRC patients using primer pair 1 although these differences also did also not reach statistical significance. Further analysis of the clinicopathologic features of patients with primary CRC with regard to NDRG4 promoter methylation for both independent series did not reveal any association with age at diagnosis, sex or the TNM stage. However, we did find a significant correlation between promoter methylation and the location of the tumor using primer pair 1 (table 49, p=0.034).

Table 49: Prevalence (%) of promoter methylation of NDRG4 in relation to clinicopathological features of carcinoma tissue for two independent series. Methylation differences were analyzed by chi-square

Characteristics		%methylation NDRG4p1	%methylation NDRG4p2	% methylation NDRG4p2 Independent series
TNM stage*				
	I	15%	16%	23%
	II	33%	32%	33%
	III	40%	41%	30%
	IV	13%	11%	13%
	P	NS	NS	NS
Tumor Location‡				
	proximal	65%	56%	37%
	distal	35%	44%	63%
	P	0.034	NS	NS
Sex*				

	Male	42%	48%	55%
	Female	58%	52%	44%
	P	NS	NS	NS
Age at diagnosis§				
	<= mean	40%	48%	48%
	> mean	60%	52%	52%
	P	NS	NS	NS

Abbreviations: P, P-value; NS, not significant

NDRG4 promoter methylation in other neoplasias

Next, we asked whether NDRG4 promoter methylation is present in other tumor tissues. Therefore 119 primary tumor specimens covering 7 different tumor types were analyzed using MSP primer pair 2. No or little methylation was found in skin (0/8, 0%), kidney (1/10, 10%), ovary (0/20, 0%), prostate (0/10, 0%) and breast (lobular (0/7, 0%) and ductal (0/9, 0%)) carcinomas. In contrast, NDRG4 promoter was frequently methylated in adenocarcinomas of the esophagus (13/16, 81%), while no methylation was found in esophageal squamous cancers (0/12, 0%). Both diffuse type (8/11, 73%) and intestinal type (9/11, 82%) carcinomas of the stomach were frequently methylated while the normal mucosa of the stomach did not show any methylation (0/5, 0%).

NDRG4 promoter methylation in fecal DNA

The high prevalence of NDRG4 promoter methylation in CRC and the absence of methylation in normal colon mucosa suggest that NDRG4 promoter methylation could be a sensitive and specific biomarker for non-invasive detection of CRC. Therefore, we developed a quantitative MSP assay using molecular beacon technology and analyzed fecal DNA of 21 CRC patients and 67 healthy controls. NDGR4 promoter methylation could be detected in 16/21 CRC patients yielding a 76%

sensitivity for the detection of CRC. Only 2/67 (3%) of healthy controls tested positive for NDRG4 methylation, which resulted in a clinical specificity for the assay of 97%. Stool samples were obtained from CRC patients covering all different TNM stages. The assay had a 75 % sensitivity among CRC patients with early stage colon cancer (stage I and II) and 80% of sensitivity among later stage patients (stage III and IV).

NDRG4 RNA and protein expression

To analyse whether methylation of the promoter CpG island of NDRG4 is associated with gene silencing we investigated mRNA expression of NDRG4 in CRC cell lines, three pairs of CRC tissues and matching normal colon mucosa. In all three CRCs, mRNA levels were significantly downregulated (97, 70% and 98% respectively) when compared to normal colon mucosa (FIG.12b).

To investigate the protein expression of NDRG4 in both normal colonic mucosa and colon cancers, we performed NDRG4 immunohistochemistry demonstrating the presence of NDRG4 protein expression in the cytoplasm of normal colon mucosa while protein expression is lost in half of CRCs (FIG.12c). Subsequently, we performed immunohistochemical analysis of NDRG4 expression on 19 CRC samples. Eleven of these patients had a methylated NDRG4 promoter. However, we could not find a significant association between NDRG4 promoter methylation and NDRG4 expression (data not shown). This observation suggests that other mechanisms might lead to NDRG4 inactivation.

Loss of heterozygosity and mutation analysis of the NDRG4 gene in CRC

Macrodissected CRC tissue and corresponding normal tissues of 86 CRC patients were analyzed using the microsatellite markers DS16S3089 and DS16S3071. The two markers showed a heterozygosity of 77.4% and 35.4% respectively. Of these, 59 cases were informative; 18 tumors (30,5%) showed LOH with at least one marker on chromosome 16q.

Twelve primary CRC and CRC cell lines HCT116 and SW480 were analyzed for NDRG4 mutations. No inactivating mutations within the coding region of the NDRG4 gene were detected in 12 colorectal carcinomas. However, we found one novel nonsynonymous mutation in the SW480 cell line (40662A→AG Ile65Val). As part of the mutational analysis, 2 previously reported SNPs (NCBI SNP database) were detected. One SNP was observed in 1/12 CRC patients (43760G→GG Val224Val refSNP rs 17821543). The second SNP was observed in 9/12 CRC patients (48311A→AG Ser354Ser refSNP rs 42945).

Discussion

The progression of CRC from small benign colorectal adenomas to larger and more dysplastic lesions takes several decades and identifying early stages would improve management and treatment of this disease (Brenner and Rennert 2005). Colonoscopy is currently the best technique for detecting CRC or its precursor lesions from the age of 50 years onwards. Testing for the presence of fecal occult blood (FOBT) as preselection for colonoscopy is the only non-invasive screening method with proven effectiveness,

reducing both the incidence and the risk of death from CRC when used programmatically.

However, both sensitivity and specificity of FOBT is low and therefore there is an urgent need for more sensitive and specific non-invasive screening tests. A promising option is analyzing (expression of) cancer-specific molecules such as DNA, RNA and protein in blood and tissue. First attempts to detect genetic alterations are promising (Dong, Traverso et al. 2001; Traverso, Shuber et al. 2002) although still need improvement. Markers of choice have been TP53, K-ras and APC mutations and in addition BAT-26 instability and long DNA (a marker for non-apoptotic shedding of epithelial colonocytes). Recently, CpG island hypermethylation can also be used as a (prognostic) marker for non-invasive detection of CRC in different biological samples (Esteller 2003; Chen, Han et al. 2005; Ebert, Model et al. 2006). Over the last years, several genes have been described to be methylated in CRC using different techniques.

Here we used MSP, quantitative MSP and bisulfite sequencing to analyse NDRG4 as a biomarker for the early detection of colorectal and other gastrointestinal cancers. (ARRAY) The NDRG4 promoter CpG island was demonstrated to be methylated in two independent large series of CRC cases. In the first series we included normal mucosa of non-cancerous patients since the normal mucosa from the CRC patients is situated within the same bowel segment as the tumor and can be contaminated with malignant cells or a field-effect could have change the molecular signature of this cell as described for MGMT (issa, 2005). Nevertheless, by performing statistical analysis we could not find any significantly difference in methylation between these two groups. Chronic

inflammation has previously been shown to accelerate DNA methylation in normal tissues (Issa, Ahuja et al. 2001). Therefore additional screens with inflamed colon mucosa are expected in a screening setting. In our study population, inclusion of inflamed mucosa to the normal mucosa of control patients slightly reduced the specificity of NDRG4 from 96% to 94%. Because we found a difference in the density of methylation in the promoter area of NDRG4 by bisulfite sequencing, we used two different primer sets to investigated the methylation status of NDRG4. Interestingly, using primer pair 2, we found 86% of methylation in carcinoma tissue while only 71% was observed by use of primer pair 1. This increased detection of methylation using primer pair 2 was observed for all the subgroups of this series as shown in table 47. Primer pair 2 is situated more to the 5'region of the gene. The frequencies of methylation were lower near the transcription start site. We hypothesize that NDRG4 hypermethylation initially occurs at the 5' end of the NDRG4 CpG island and spreads towards the transcription start site before ultimately shutting down NDRG4 mRNA expression, as has also been observed for RUNX3 (Turker 2002; Homma, Tamura et al. 2006). In addition, we found a significant difference (p=0.012) in methylation frequency using primer pair 1, between adenoma tissue and carcinoma tissue within the group of CRCs. Therefore, we speculated, that spreading of DNA methylation in the promoter area of NDRG4 towards the transcription start site occurs during cancer progression.

Remarkably, using primer pair 1, hypermethylation was more frequently present in progressed adenomas from the CRC patients (41%) when compared to the non progressing adenomas

of the CRC- patients (13%). The capacity to distinguish adenomas that progress to cancer from those that will not progress is highly important for CRC screening (Hermsen, Postma et al. 2002). Whereas this difference can not be made macroscopically, endoscopic screening strategies aiming to detect and remove all adenomas will be inherently unspecific. The majority of adenomas removed would not have progressed to cancer because only a small percentage of these benign precursor lesions will progress into a carcinoma (Lengauer, Kinzler et al. 1998). These data might indicate that NDRG4 promoter methylation in adenoma tissue (in the region we investigated) is a possible risk factor for developing a colon tumor.

Recently, it has been reported that promoter methylation can be detected in biological fluids such as blood, urine or stool and may allow early diagnosis of various cancers, including CRC. Some studies have shown that methylation of one gene promoter can be used as a screening method for fecal DNA methylation detection. For example, promoter methylation of SFR2, Vimentin and HIC1 can be detected in fecal DNA of CRC patients with a sensitivity of 77%, 43% and 42% respectively and a specificity of 77%, 90% and 95% respectively (Muller, Oberwalder et al. 2004; Chen, Han et al. 2005; Lenhard, Bommer et al. 2005). NDRG4 methylation in fecal DNA as a single marker can differentiate cancer from controls with a sensitivity of 76% and a specificity of 97%.

In order to be a specific biomarker for CRC, analysis of tissue specificity was performed; we found NDRG4 methylation in other tumors of the gastrointestinal tract, namely oesophagus and gastric cancers. This data indicate that

methylation of NDRG4 may serve as a marker for other gastrointestinal tumors as well.

We next studied whether methylation of NDRG4 is associated with downregulation of NDRG4 RNA and protein expression. So far, the expression of NDRG4 has only been documented in the brain and heart by use of Northern blotting. We observed expression of NDRG4 in normal colon tissue and downregulation in al three tumor tissues. Subsequently, we performed immunohistochemical analysis of NDRG4 expression on 19 CRC samples from the CRC patients for which paraffin-embedded tissues were available. Eleven of these patients had a methylated NDRG4 promoter. However, we could not find a significant association between NDRG4 promoter methylation and NDRG4 expression. Some tumors had a methylated NDRG4 promoter although still expressed NDRG4 protein. The methylation that we detected using MSP might reflect methylation of only a few cancer cells or methylation of only one of two NDRG4 alleles (and absence in the other). Nevertheless, some tumors lack expression of NDRG4 protein while no promoter methylation was observed. This observation suggests that other mechanisms might lead to NDRG4 inactivation. No mutations were found, indicating that mutational inactivation of the NDRG4 gene might not play a mayor role in CRC. Our results confirmed previous data on NDRG4 mutation studies (Sjoblom, Jones et al. 2006). However, LOH at 16q is seen in about 30% of the CRC cases. Frequent LOH of 16q had previously been described in a wide variety of solid tumor types as breast (Rakha, Green et al. 2006), liver (Sakai, Nagahara et al. 1992; Bando, Nagai et al. 2000), prostate (Elo, Harkonen et al. 1997), ovarian (Kawakami, Staub et al. 1999) and Wilms' tumors (Mason,

Goodfellow et al. 2000) but until now it has not been described in CRC. Because NDRG4 is downregulated in most of the colon cancer cells compared to normal colonic epithelial cells we hypotheses that NDRG4 has a tumor suppressor function in cancer.

In conclusion, we are the first group who described a role for NDRG4 in cancer and our data indicate that NDRG4 is a potential novel marker for CRC with a very high sensitivity and specificity of 76% and 100% respectively. Although the sensitivity and specificity of NDRG4 as a marker alone is already very high, the diagnostic accuracy of NDRG4 may be enhanced by the addition of other markers analyzed in patients with CRC as well. This may augment the ability to identify patients with cancer in a multipanel methylation-based diagnostic test.

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5) Additional real-time MSP assays tested on plasmid material for NDRG4 and OSMR genes

Plasmid material corresponding to a promoter region of NDRG4 and OSMR gene was used to test additional assay designs. The plasmid for the standard curve was generated as follows: the promoter sequence as defined by the primers is PCR amplified and cloned (using suitable isolated and bisulphite modified cell line DNA). The sequence is verified by sequencing and compared to the published promoter sequence. A serial dilution of either NDRG4 or OSMR plasmid material ($2x10^6$ to $2x10^1$copies/5µl) was loaded in duplicate. 5 µl of plasmid dilution or buffer (non template control) was added to a 20 µl PCR mix containing the specified primer and beacon detector sequences as previously described. Results were generated using the SDS 2.2 software from Applied Biosystems with automatic baseline and threshold settings. Data were exported as Ct values (cycle number at which the amplification curves cross the threshold value, set automatically by the software).

NDRG4

Initial real-time results for 2 different NDRG4 assay designs are presented in table 50 and 51. The primer and beacon combinations used for the respective assays NDRG4_1a and NDRG4_1b were previously described. Underscore 1a and 1b reflect the different primer and/or beacon combinations used for assessing the methylation status of the NDRG4 gene. NDRG4_1a corresponds to the preferred NDRG4 assay design, also simply referred to as NDRG4 (see Table 4). Comparable results were obtained for both assay designs. Clinical

sample data provided in this invention are generated using the preferred NDRG4 assay design (=NDRG4 = NDRG4_1a)

Table 50: Real time MSP results obtained for NDRG4_1a assay on plasmid material. Resulting standard curve (y = -3.3321x + 39.862; R2 = 0.9991) corresponds to a PCR efficiency of 100%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
NDRG4_1a	Standard	18.82	2000000	6.30	18.92	18.87	0.09
NDRG4_1a	Standard	22.09	200000	5.30	22.22	22.15	0.13
NDRG4_1a	Standard	25.42	20000	4.30	25.47	25.45	0.06
NDRG4_1a	Standard	28.86	2000	3.30	28.94	28.90	0.08
NDRG4_1a	Standard	32.58	200	2.30	32.48	32.53	0.10
NDRG4_1a	Standard	34.92	20	1.30	35.64	35.28	0.71
NDRG4_1a	NTC	Undetermined	0		Undetermined	Undeter.	Undeter.

Table 51: Real time MSP results obtained for NDRG4_1b assay on plasmid material. Resulting standard curve (y = -3.4181x + 40.991; R2 = 0.9991) corresponds to a PCR efficiency of 99.2%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
NDRG4_1b	Standard	19.48	2000000	6.30	19.59	19.53	0.12
NDRG4_1b	Standard	22.93	200000	5.30	22.92	22.92	0.01
NDRG4_1b	Standard	26.26	20000	4.30	26.18	26.22	0.08
NDRG4_1b	Standard	29.65	2000	3.30	29.67	29.66	0.02
NDRG4_1b	Standard	32.82	200	2.30	32.83	32.82	0.01
NDRG4_1b	Standard	36.75	20	1.30	36.91	36.83	0.16
NDRG4_1b	NTC	Undetermined	0		Undetermined	Undet	Undet

OSMR

Initial real-time results for 3 different OSMR assay designs are presented in below Tables 52 to 54. The primer and beacon combinations used for the respective assays OSMR_1, OSMR_3 [=OMSR (3)] and OSMR_4 [=OSMR (4)] were previously described. Underscore 1, 3 and 4 reflect the different primer and/or beacon combinations used for assessing the methylation status of the OSMR gene. Comparable results were obtained for all three assay designs.

Table 52: Real time MSP result obtained for OSMR_1 assays on plasmid material. Resulting standard curve (y = -3.3326x + 41.136; R2 = 0.9993) corresponds to a PCR efficiency of 99.6%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
OSMR_1	Standard	20.04	2000000	6.30	20.14	20.09	0.09
OSMR_1	Standard	23.48	200000	5.30	23.41	23.44	0.07
OSMR_1	Standard	26.73	20000	4.30	26.85	26.79	0.12
OSMR_1	Standard	30.13	2000	3.30	30.26	30.19	0.13
OSMR_1	Standard	33.55	200	2.30	33.93	33.74	0.38
OSMR_1	Standard	36.54	20	1.30	36.58	36.56	0.04
OSMR_1	NTC	Undetermined	0		Undetermined	Undet	Undet

Table 52: Real time MSP result obtained for OSMR_3 assays on plasmid material. Resulting standard curve (y = -3.3909x + 38.398; R2 = 0.9999) corresponds to a PCR efficiency of 97.2%.

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
OSMR_3	Standard	16.93	2000000	6.30	17.16	17.04	0.23
OSMR_3	Standard	20.41	200000	5.30	20.29	20.35	0.12
OSMR_3	Standard	23.97	20000	4.30	23.83	23.90	0.14
OSMR_3	Standard	27.22	2000	3.30	27.16	27.19	0.06
OSMR_3	Standard	30.51	200	2.30	30.67	30.59	0.16
OSMR_3	Standard	34.18	20	1.30	33.77	33.98	0.41
OSMR_3	NTC	38.13	0		Undetermined	Undet	Undet

Table 54: Real time MSP result obtained for OSMR_4 assays on plasmid material. Resulting standard curve (y = -3.2795x + 38.77; R2 = 0.9997) corresponds to a PCR efficiency of 100.8%

Assay	Task	Ct	Quantity	Log copies	Duplicate Ct	Average Ct	ΔCt
OSMR_4	Standard	18.24	2000000	6.30	17.90	18.07	0.33
OSMR_4	Standard	21.56	200000	5.30	21.05	21.31	0.51
OSMR_4	Standard	24.79	20000	4.30	24.64	24.72	0.15
OSMR_4	Standard	28.24	2000	3.30	27.91	28.08	0.33
OSMR_4	Standard	31.37	200	2.30	31.18	31.28	0.19
OSMR_4	Standard	34.63	20	1.30	34.12	34.37	0.50
OSMR_4	NTC	Undetermined	0		Undetermined	Undet	Undet

6) **Testing and validation of further CRC markers in bodily fluid test samples**

New markers added: BNIP3, FOXE1, JAM3, PHACTR3, TPFI2, SOX17 and SYNE1 (and also JPH3 stool data). Suitable primers and probes for determining the methylation status of these genes are set forth in Tables 12 (and 13 to 18) above.

Methods and Results

Clinical samples

Samples were collected from centers in Germany and The Netherlands

Table 55: Samples for DNA extraction from plasma (blood origin)

type	Sample Numbers
Normal	10
Colorectal Cancer stage III	6
Colorectal Cancer stage IV	4

Table 56: Samples for DNA extraction from stool

type	Sample Numbers
Control (Normal)	7
Case (Colorectal Cancer)	1
Case (Colorectal Cancer)	6

Marker testing on clinical samples

Experiments were performed as previously described. Briefly DNA was extracted from stool and/or plasma followed by bisulfite treatment. Samples were tested by real-time MSP assays, using 384 well plates with a 12 µl final volume. The template volume is 2.4 µl with a mix volume of 9.6 µl.

Results were generated using the SDS 2.2 software (Applied Biosystems), exported as Ct values (cycle number at which the amplification curves cross the threshold value, set automatically by the software). Copy numbers are extrapolated using a standard curve.

The individual performance of the 8 gene assays TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, JAM3 and JPH3 in plasma and stool samples is shown in Table XV (except for JPH3: stool data only). Sensitivity values for plasma and stool are ranging from 30 to 70% and 0 to 57% respectively with a corresponding specificity of a 100%. When optimizing for sensitivity, 80% sensitivity for TFPI2 and 50% sensitivity for PHACTR3 is obtained in plasma samples with a corresponding specificity of 90%. It is observed that for some markers (TFPI2, BNIP3, FOXE1, SYNE1 and SOX17) sensitivity of colorectal cancer detection is higher when using plasma samples compared to stool samples.

Table 57: Individual gene performance: Sensitivity and specificity of TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, JAM3 markers on stool and plasma samples. Sensitivity and specificity results for the JPH3 marker were only obtained for stool using this sample set, plasma data were enabled earlier with a different sample set.

	optimized for Specificity			optimized for Sensitivity		
	Specificity	Sensitivity	cutoff	Specificity	Sensitivity	cutoff
TFPI2 Stool	100	57	10			
TFPI2 Plasma	100	70	1	90	80	0
BNIP3 Stool	100	0	7			
BNIP3 Plasma	100	30	0			
FOXE1 Stool	100	57	0			
FOXE1 Plasma	100	60	0			

SYNE1 Stool	100	57	2			
SYNE1 Plasma	100	60	0			
SOX17 Stool	100	57	30			
SOX17 Plasma	100	60	2			
PHACTR3 Stool	100	43	8			
PHACTR3 Plasma	100	40	2	90	50	0
JAM3 Stool	100	43	1			
JAM3 Plasma	100	30	1			
JPH3 Stool	100	14	20			
JPH3 Plasma	see previous colon results					

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

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

1. A method of detecting a predisposition to, or the incidence of, cancer in a sample comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of a predisposition to, or the incidence of, cancer.

2. The method of claim 1 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

3. The method of claim 1 or 2 wherein the test sample is a faecal test sample.

4. The method of claim 1 which comprises detecting epigenetic silencing of an NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the gene is indicative for cancer or predisposition to cancer or advanced adenoma.

5. The method according to claim 1 wherein NDRG4/NDRG2 subfamily gene expression is determined, wherein reduced expression of the gene is indicative for colon cancer and/or gastric cancer, or predisposition to colon cancer and/or gastric cancer, or advanced adenoma.

6. The method according to claim 1 wherein NDRG4/NDRG2 subfamily gene methylation status is determined, wherein

methylation of the gene is indicative for colon cancer and/or gastric cancer, or predisposition to colon cancer and/or gastric cancer, or advanced adenoma.

7. The method of claim 1 wherein the cancer is colorectal cancer, the sample is a blood sample, or derivative thereof and the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

8. The method of claim 1 wherein the cancer is colorectal cancer, the sample is a tissue sample and the at least one gene is a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

9. The method of claim 1 wherein the cancer comprises a gastrointestinal cancer.

10. The method of claim 9 wherein the gastrointestinal cancer comprises colorectal cancer, gastric cancer, stomach cancer and/or oesophageal cancer.

11. The method of claim 10 wherein the gastrointestinal cancer is colorectal cancer.

12. The method of claim 10 wherein the oesophageal cancer is oesophageal adenocarcinoma.

13. The method of claim 10 wherein the stomach cancer comprises a diffuse type and/or intestinal type carcinoma of the stomach.

14. The method of any preceding claim wherein the at least one gene is an NDRG4/NDRG2 subfamily gene.

15. The method of any preceding claim wherein the NDRG4/NDRG2 subfamily gene comprises or consists of the NDRG4 gene.

16. The method of any of claims 1 to 13 wherein the at least one gene is selected from GATA4, OSMR, NDRG4 and SFRP2.

17. The method of any of claims 1 to 13 wherein the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23.

18. The method of any preceding claim which comprises detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, cancer.

19. The method of claim 18 wherein the panel of genes comprises two, three, four, five or six genes.

20. The method of claim 18 or 19 wherein the panel of genes comprises GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2, NDRG4 and SFRP2, APC and

SFRP2, APC and OSMR, APC and GATA4, APC and NDRG4, MGMT and OSMR, MGMT and GATA4, MGMT and NDRG4, MGMT and SFRP2, MGMT and APC, SFRP1 and MGMT, SFRP1 and OSMR, SFRP1 and GATA4, SFRP1 and NDRG4, SFRP1 and SFRP2, SFRP1 and APC, GATA5 and SFRP1, GATA5 and MGMT, GATA5 and OSMR, GATA5 and GATA4, GATA5 and NDRG4, GATA5 and SFRP2 or GATA5 and APC.

21. The method of any of claims 18 to 20 wherein the panel of genes comprises GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2, GATA4, NDRG4 and SFRP2, SFRP1, SFRP2 and APC, SFRP2, OSMR and APC or OSMR, NDRG4 and SFRP2.

22. The method of any of claims 18 to 21 wherein the panel of genes consists of GATA4, OSMR, NDRG4 and SFRP2.

23. The method of any of claims 18 to 20 wherein the panel of genes comprises NDRG4, OSMR, SFRP1, ADAM23, GATA5, GATA4 and MGMT.

24. The method of claim 18 or 19 wherein the panel of genes is a three gene panel with the genes being selected from OSMR, NDRG4, GATAJ and ADAM23.

25. The method of claim 24 wherein the panel of genes consists of OSMR, NDRG4, GATA5 and ADAM23.

26. The method of claim 18 or 19 wherein the panel of genes comprises OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5.

27. The method of any of claims 18 to 20 wherein the detection of an epigenetic change in each of the panel of genes is carried out in a single reaction.

28. The method of any preceding claim wherein the sample comprises a tissue sample and/or a bodily fluid sample.

29. The method of claim 28 wherein the bodily fluid sample comprises a faecal sample.

30. The method of claim 29 wherein the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT and/or selected from TFPI2, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

31. The method of claim 28 wherein the tissue sample comprises a colon and/or rectal and/or appendix sample.

32. The method of claim 28 wherein the bodily fluid sample comprises a blood sample.

33. The method of claim 32 wherein the blood sample, or derivative thereof comprises a plasma or a serum sample.

34. The method of claim 32 or 34 wherein the at least one gene is selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3, JPH3 and JAM3.

35. The method of claim 33 or 34 wherein the plasma sample is obtained by centrifugation of whole blood.

36. The method of claim 35 wherein multiple centrifugation steps are employed to obtain the plasma sample.

37. The method of claim 36 wherein two centrifugation steps are employed to obtain the plasma sample.

38. The method of claim 37 wherein plasma samples of volume of less than 2 ml are excluded.

39. The method of any preceding claim wherein the cancer is colorectal cancer and wherein the colorectal cancer comprises early stage colorectal cancer.

40. The method of claim 39 wherein the early stage colorectal cancer comprises a stage 0-II colorectal cancer.

41. The method of any preceding claim wherein the epigenetic change is methylation.

42. The method of claim 41 wherein hypermethylation of the promoter region of the gene(s) is detected.

43. The method of claim 41 or 42 wherein methylation is determined using methylation specific PCR/amplification.

44. The method of claim 43 wherein the methylation specific PCR/amplification is carried out in real time or at end point.

45. The method of any of claims 41 to 44 wherein methylation is quantified against methylation of a reference gene.

46. The method of any of claims 43 to 44 which utilises primers selected from primers comprising the nucleotide sequences set forth in tables 2 to 18.

47. The method of any of claims 43 to 46 which utilises probes selected from probes comprising the nucleotide sequences set forth in tables 2 to 18.

48. The method of any preceding claim which is used in combination with detecting DNA integrity, or at least one DNA oncogene mutation, or a combination of both detecting DNA integrity and at least one DNA oncogene mutation in the sample in order to detect a predisposition to, or the incidence of, colorectal cancer.

49. The method of any preceding claim wherein the methylation status of the CpG island comprising the nucleotide sequence set forth as SEQ ID NO: 524 and/or SEQ ID NO: 525 is determined.

50. The method of any preceding claim wherein the epigenetic change is detected by determination of its effect at the level of gene expression.

51. The method of claim 50 wherein gene expression is determined at the protein level.

52. The method of claim 50 wherein gene expression is determined at the RNA level.

53. The method of claim 52 wherein real time or end-point detection is employed.

54. A method for determining the histopathological stage of cancer and in particular a gastrointestinal cancer in a sample comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative of the histopathological stage of the cancer.

55. The method of claim 54 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

56. The method of claim 54 or 55 wherein the test sample is a faecal sample.

57. The method of claim 51 which is for determining the histopathological stage of adenoma and/or colorectal cancer and comprises determining epigenetic silencing of the NDRG4/NDRG2 subfamily gene, wherein epigenetic silencing of the NDRG2/NDRG4-family gene is correlated to a histopathological stage of the cancer.

58. The method of claim 54 which is for determining the histopathological stage of colorectal cancer in a blood sample, or derivative thereof and comprises detecting an epigenetic change in at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic

change is indicative of the histopathological stage of the colorectal cancer.

59. The method of claim 54 which is for determining the histopathological stage of colorectal cancer in a tissue sample and comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative of the histopathological stage of the colorectal cancer.

60. A method for predicting or monitoring progression of an adenoma to a carcinoma comprising determining the methylation status of an NDRG2/NDRG4 subfamily gene in a suitable test sample, wherein an elevated or increased level of methylation indicates that the adenoma is more likely to progress to a carcinoma than if the level of methylation is lower.

61. The method of claim 60 wherein the methylation status of the NDRG4 gene is determined.

62. The method of claim 61 wherein the methylation status of the NDRG4 gene in the region between and including the primer binding sites of the primers comprising the nucleotide sequences set forth in table 2 is determined.

63. A method for predicting or monitoring progression of a gastrointestinal cancer comprising determining the methylation status of the NDRG4 gene in a suitable test sample, wherein an elevated or increased level of methylation towards the transcription start site indicates

that the cancer is more progressed than if the level of methylation is lower.

64. The method of claim 63 wherein the gastrointestinal cancer is colorectal cancer.

65. A method for predicting the likelihood of successful treatment of cancer and in particular a gastrointestinal cancer with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

66. The method of claim 65 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

67. The method of claim 65 or 66 wherein the test sample is a faecal sample.

68. The method of claim 65 which comprises determining the methylation status of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the NDRG4/2 family gene is methylated, in particular hypermethylated, the likelihood of successful treatment is higher than if the NDRG4/2 family gene is unmethylated, or methylated to a lesser degree.

69. The method of claim 65 which comprises measurement of expression levels of the NDRG4/2 family gene in a sample obtained from a subject, wherein a reduced level of expression indicates the likelihood of successful treatment of cancer is higher than if the NDRG4/2 family gene is expressed at a higher level.

70. The method of claim 65 wherein the cancer is colorectal cancer, the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or selected from TFPI1, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 and the sample is a blood sample, or derivative thereof.

71. The method of claim 65 wherein the cancer is colorectal cancer and the method comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5 in a tissue sample, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

72. A method for predicting the likelihood of resistance to treatment of cancer and in particular a gastrointestinal cancer with a DNA demethylating agent and/or DNA methyltransferase inhibitor and/or HDAC inhibitor comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change is indicative that the likelihood of

resistance to treatment is lower than if the epigenetic modification is not detected.

73. The method of claim 72 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

74. The method of claim 72 or 73 wherein the test sample is a faecal sample.

75. The method of claim 72 which comprises determining the methylation status of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the NDRG4/2 family gene is unmethylated, or methylated to a lesser degree, the likelihood of resistance to treatment is higher than if the NDRG4/2 family gene is methylated or hypermethylated.

76. A method of claim 72 which comprises measurement of expression levels of the NDRG4/2 family gene in a sample obtained from a subject, wherein a higher level of expression indicates the likelihood of resistance to treatment of cancer is higher than if the NDRG4/2 family gene is expressed at a reduced level.

77. The method of claim 72 wherein the cancer is colorectal cancer, the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 and the sample is a blood sample, or derivative thereof.

78. The method of claim 72 wherein the cancer is colorectal cancer and the method comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5 in a tissue sample, wherein detection of the epigenetic change in at least one of the genes in the panel is indicative that the likelihood of successful treatment is higher than if the epigenetic modification is not detected.

79. A method of selecting a suitable treatment regimen for cancer in particular a gastrointestinal cancer comprising detecting an epigenetic change in at least one gene selected from an NDRG4/NDRG2 subfamily gene, GATA4, OSMR, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC, MGMT, TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3, wherein detection of the epigenetic change results in selection of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor for treatment and wherein if the epigenetic change is not detected, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment.

80. The method of claim 79 wherein the cancer is colorectal cancer and the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

81. The method of claim 79 or 80 wherein the sample is a faecal sample.

82. The method of claim 79 which comprises determining epigenetic silencing of a NDRG4/2 family gene in a sample

obtained from a subject, wherein if the gene is epigenetically silenced, in particular hypermethylated or reduced expressed, a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is selected for treatment.

83. The method of claim 79 which is for selecting a suitable treatment regimen for cancer or predisposition to cancer or advanced adenoma and comprises determining the methylation status and/or expression level of a NDRG4/2 family gene in a sample obtained from a subject, wherein if the gene is unmethylated or higher expressed, treatment with a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor is contra-indicated.

84. The method of claim 79 wherein the cancer is colorectal cancer, the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC and/or is selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3 and the sample is a blood sample, or derivative thereof.

85. The method of claim 79 wherein the cancer is colorectal cancer and the method comprises detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5 in a tissue sample, wherein detection of the epigenetic change in at least one of the genes in the panel results in selection of a DNA demethylating agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor for treatment and wherein if the epigenetic change in at least one of the genes in the panel is not detected, a DNA demethylating agent and/or a DNA

methyltransferase inhibitor and/or a HDAC inhibitor is not selected for treatment.

86. The method of any one of claims 54 to 85 further characterised by the features of the method as claimed in any of claims 1 to 53.

87. A method of treating cancer in a subject comprising administration of a DNA demethylating agent and/or a HDAC inhibitor and/or a DNA methyltransferase inhibitor wherein the subject has been selected for treatment on the basis of a method as claimed in any preceding claim.

88. The method of claim 87 wherein the subject has been selected for treatment on the basis of measuring the methylation status of the NDRG2/NDRG4-family gene promoter, the expression level of the NDRG2/NDRG4-family gene, or a combination thereof.

89. The method of claim 87 or 88 wherein the cancer comprises a gastrointestinal cancer.

90. The method of claim 89 wherein the gastrointestinal cancer comprises colorectal cancer, gastric cancer, stomach cancer and/or oesophageal cancer, in particular colorectal cancer.

91. The method of claim 90 wherein the oesophageal cancer is oesophageal adenocarcinoma.

92. The method of claim 90 wherein the stomach cancer comprises a diffuse type and/or intestinal type carcinoma of the stomach.

93. A kit for;
(a) predicting the likelihood of successful treatment of cancer and/or the likelihood of resistance to treatment of cancer with a DNA damaging agent and/or a DNA methyltransferase inhibitor and/or a HDAC inhibitor,
(b) for selecting a suitable treatment regimen for cancer
(c) for diagnosing cancer or predisposition to cancer,
comprising carrier means containing therein a set of primers for use in detecting the methylation status of at least one gene selected from a NDRG2/NDRG4-family gene, GATA4, OSMR, GATA5, SERP1, ADAM23, JPH3, SFRP2, APC and MGMT, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

94. The kit of claim 93 wherein the at least one gene is a NDRG2/NDRG4 subfamily gene.
95. The kit of claim 94 wherein the NDRG2/NDRG4 subfamily gene is NDRG4.

96. The kit of any of claims 93 to 95 which further comprises a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues.

97. The kit of claim 96 wherein the reagent comprises a bisulphite reagent.

98. The kit of claim 97 wherein the bisulphite reagent comprises sodium bisulphite.

99. A kit for detecting a predisposition to, or the incidence of a gastrointestinal cancer and in particular colorectal cancer in a sample comprising:
(a) means for detecting an epigenetic change in at least one gene selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3
(b) means for processing a faecal sample.

100. The kit of any of claims 93 to 99 wherein the at least one gene is selected from GATA4, OSMR, NDRG4, GATA5, SFRP1, ADAM23, JPH3, SFRP2, APC and MGMT.

101. The kit of claim 93 wherein the at least one gene is selected from GATA4, OSMR, NDRG4 and SFRP2.

102. The kit of any of claims 93 to 101 which comprises means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

103. The kit of claim 102 wherein the panel of genes comprises two, three, four, five or six genes.

104. The kit of claim 102 or 103 wherein the panel of genes comprises GATA4 and OSMR, GATA4 and NDRG4, GATA4 and SFRP2, OSMR and NDRG4, OSMR and SFRP2 or NDRG4 and SFRP2.

105. The kit of any of claims 102 to 104 wherein the panel of genes comprises GATA4, OSMR and NDRG4, GATA4, OSMR and SFRP2, GATA4, NDRG4 and SFRP2 or OSMR, NDRG4 and SFRP2.

106. The kit of any of claims 102 to 105 wherein the panel of three or four genes selected from genes consists of GATA4, OSMR, NDRG4 and SFRP2.

107. The kit of claim 102 or 103 wherein the panel of genes comprises NDRG4, OSMR, SFRP1, ADAM23, GATA5 and MGMT.

108. The kit of any of claims 99 to 107 wherein the means for processing a faecal sample comprise a sealable vessel for collection of a faecal sample.

109. The kit of any of claims 99 to 108 wherein the means for processing a faecal sample comprises a homogenization buffer.

110. The kit of any of claims 99 to 109 wherein the means for processing a faecal sample comprises reagents for extraction/isolation/concentration/purification of DNA.

111. The kit of any of claims 99 to 110 wherein the means for processing a faecal sample comprises primers for directing amplification of DNA in the sample.

112. A kit for detecting a predisposition to, or the incidence of, gastrointestinal cancer and in particular colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in at least one gene selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC, and/or at least one gene selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3

(b) means for processing a blood sample, or derivative thereof.

113. The kit of claim 112 wherein the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

114. The kit of claim 112 or 113 wherein the blood sample, or derivative thereof comprises a plasma or a serum sample.

115. The kit of any of claims 112 to 114 wherein the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23.

116. The kit of any of claims 112 to 115 which comprises means for detecting an epigenetic change in a panel of genes comprising at least two, three, four, five or six of the genes, wherein detection of an epigenetic change in at least one of the genes in the panel is indicative of a predisposition to, or the incidence of, colorectal cancer.

117. The kit of claim 116 wherein the panel of genes comprises two, three, four, five or six genes.

118. The kit of claim 116 or 117 wherein the panel of genes consists of OSMR, NDRG4, GATA5 and ADAM23.

119. The kit of any of claims 112 to 118 wherein the colorectal cancer comprises early stage colorectal cancer.

120. The kit of claim 119 wherein the early stage colorectal cancer comprises a stage 0-II colorectal cancer.

121. The kit of any of claims 112 to 120 wherein the means for processing a blood sample or derivative thereof comprise a sealable vessel for collection of a blood sample.

122. The kit of any of claims 112 to 121 wherein the means for processing a blood sample, or derivative thereof comprises reagents for extraction/isolation/concentration/purification of DNA from a blood sample, or derivative thereof.

123. The kit of claim 122 wherein the reagents for extraction/isolation/concentration/purification of DNA from a blood sample, or derivative thereof are selected from the kits of table 1.

124. The kit of any of claims 112 to 123 wherein the means for processing a blood sample, or derivative thereof comprises a stabilizer.

125. A kit for detecting a predisposition to, or the incidence of, colorectal cancer in a sample comprising:

(a) means for detecting an epigenetic change in a panel of genes comprising OSMR, GATA4 and ADAM23 or OSMR, GATA4 and GATA5

(b) means for processing a colon, rectal or appendix tissue sample.

126. The kit of any of claims 93 to 125 wherein the means for detecting an epigenetic change in the panel of genes enable the detection to be carried out in a single reaction.

127. The kit of any of claims 93 to 126 wherein the epigenetic change is methylation.

128. The kit of claim 127 wherein hypermethylation of the promoter region of the gene(s) is detected.

129. The kit of claim 127 or 128 wherein the means for detecting methylation comprises methylation specific PCR primers.

130. The kit of claim 129 further comprising means for carrying out the methylation specific PCR in real time or at end point.

131. The kit of claim 129 or 130 wherein the methylation specific PCR primers are selected from primers comprising the nucleotide sequences set forth in tables 2 to 18.

132. The kit of claim 130 or 131 which comprises probes selected from probes comprising the nucleotide sequences set forth in tables 2 to 18.

133. The kit of any of claims 99 to 132 wherein the means for processing a faecal sample or blood sample, or derivative thereof or a tissue sample comprises a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues.

134. The kit of claim 133 wherein the reagent comprises a bisulphite reagent.

135. The kit of claim 134 wherein the bisulphite reagent comprises sodium bisulphite.

136. A method of determining the methylation status of at least one gene in a blood plasma or serum sample, comprising:

(a) isolating DNA from a blood plasma or serum sample,

(b) subjecting the isolated DNA to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues

(c) amplifying the treated isolated DNA in order to determine the methylation status of at least one gene, characterised in that 0.07 to 0.72 ml blood plasma or serum sample equivalent of DNA is used per amplification reaction.

137. The method of claim 136 wherein the blood plasma or serum sample is a blood plasma sample.

138. The method of claim 137 wherein the blood plasma sample is obtained by centrifugation of whole blood.

139. The method of claim 138 wherein multiple centrifugation steps are employed to obtain the plasma sample.

140. The method of claim 1381 wherein two centrifugation steps are employed to obtain the plasma sample.

141. The method of any of claims 136 to 140 wherein samples with a plasma volume less than 2 ml prior to isolating DNA are excluded.

142. The method of any of claims 136 to 141 wherein the reagent comprises a bisulphite reagent.

143. The method of any of claims 136 to 142 wherein the volume of the blood sample, or derivative thereof is around 10 ml.

144. The method of any of claims 136 to 143 further comprising following step (b) concentrating the treated isolated DNA prior to amplification.

145. The method of any of claims 136 to 143 further comprising stabilization of the blood plasma or serum sample with a stabilizer.

146. The method of any of claims 136 to 145 wherein the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2, APC and, MGMT and/or is selected from TFPI2, BNIP3, FOXE1, SYNE1, SOX17, PHACTR3 and JAM3.

147. The method of claim 146 wherein the at least one gene is selected from OSMR, SFRP1, NDRG4, GATA5, ADAM23, JPH3, SFRP2 and APC.

148. The method of claim 147 wherein the at least one gene is selected from OSMR, NDRG4, GATA5 and ADAM23.

149. The method of any of claims 136 to 148 wherein the at least one gene forms part of a panel of genes comprising at least two, three, four, five or six genes, wherein the methylation status of each of the genes is determined.

150. The method of claim 149 wherein the panel of genes comprises two, three, four, five or six genes.

151. The method of claim 149 or 150 wherein the panel of genes consists of OSMR, NDRG4, GATA5 and ADAM23.

152. The method of any of claims 149 to 151 wherein the determination of the methylation status of each of the panel of genes is carried out in a single reaction.

153. The method of any preceding claim wherein the amplifying step comprises the polymerase chain reaction (PCR).

154. The method of claim 153 wherein the PCR is methylation specific PCR.

155. The method of claim 154 wherein the methylation specific PCR is carried out in real-time or at end point.

156. The method of any of claims 136 to 155 which employs primers and/or probes selected from the primers and probes comprising the nucleotide sequences set forth in tables 2 to 18 to determine the methylation status of the at least one gene.

157. The method of any of claims 136 to 156 wherein the methylation status of the at least one gene is correlated with the incidence of a disease and the method is used in order to detect a predisposition to, or the incidence of, the disease.

158. The method of claim 157 wherein the disease comprises a cell proliferative disorder.

159. The method of claim 158 wherein the cell proliferative disorder comprises cancer.

160. The method of claim 159 wherein the cancer comprises a gastrointestinal cancer.

161. The method of claim 160 wherein the gastrointestinal cancer comprises a colorectal cancer.

162. The method of claim 161 wherein the colorectal cancer comprises early stage colorectal cancer.

163. The method of claim 162 wherein the early stage colorectal cancer comprises a stage 0-II colorectal cancer.

164. A method of processing a faecal sample to isolate and prepare DNA for use in detecting a predisposition to, or the incidence of, colorectal cancer in a faecal sample comprising:

(a) isolating DNA from the faecal sample

(b) subjecting at least 2.5µg of the isolated DNA per amplification reaction required to treatment with a reagent which selectively modifies unmethylated cytosine residues in the DNA contained in the sample to produce detectable modified residues but which does not modify methylated cytosine residues

(c) amplifying the treated isolated DNA.

165. The method of claim 164 which comprises, prior to step (a), adding a homogenization buffer to the faecal sample.

166. The method of claim 164 or 165 wherein the reagent comprises a bisulphite reagent.

167. The method of claim 166 wherein the bisulphite reagent comprises sodium bisulphite.

168. The method of any of claims 5164 to 167 wherein following step (b) and prior to step (c) the treated isolated DNA is concentrated.

169. The method of any of claims 164 to 168 wherein the faecal sample is at least 4g in weight.

170. The method of any of claims 164 to 169 further comprising carrying out the method of any of claims 1 to 58 on the amplified treated DNA.

171. A primer pair selected from the primer pairs comprising the nucleotide sequences set forth in tables 2 to 18.

172. A probe selected from the probes comprising the nucleotide sequences set forth in tables 2 to 18.

173. A kit comprising a primer pair of claim 171 and corresponding probe of claim 172.

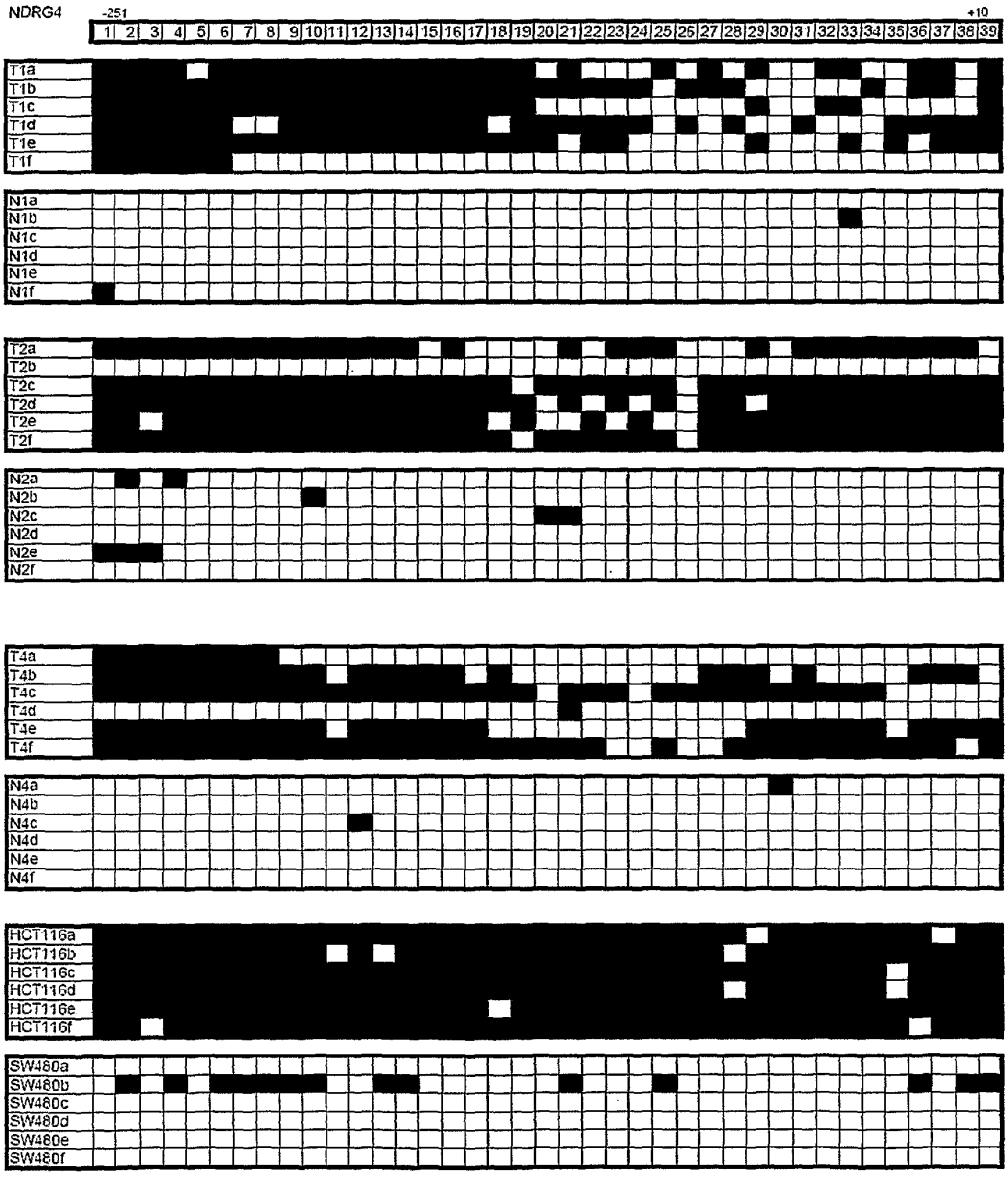

NDRG4
-251
+10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
T1a
T1b
T1c
T1d
T1e
T1f
N1a
N1b
N1c
N1d
N1e
N1f
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T4f
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N4e
N4f
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HCT116b
HCT116c
HCT116d
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SW480f

FIG. 1a

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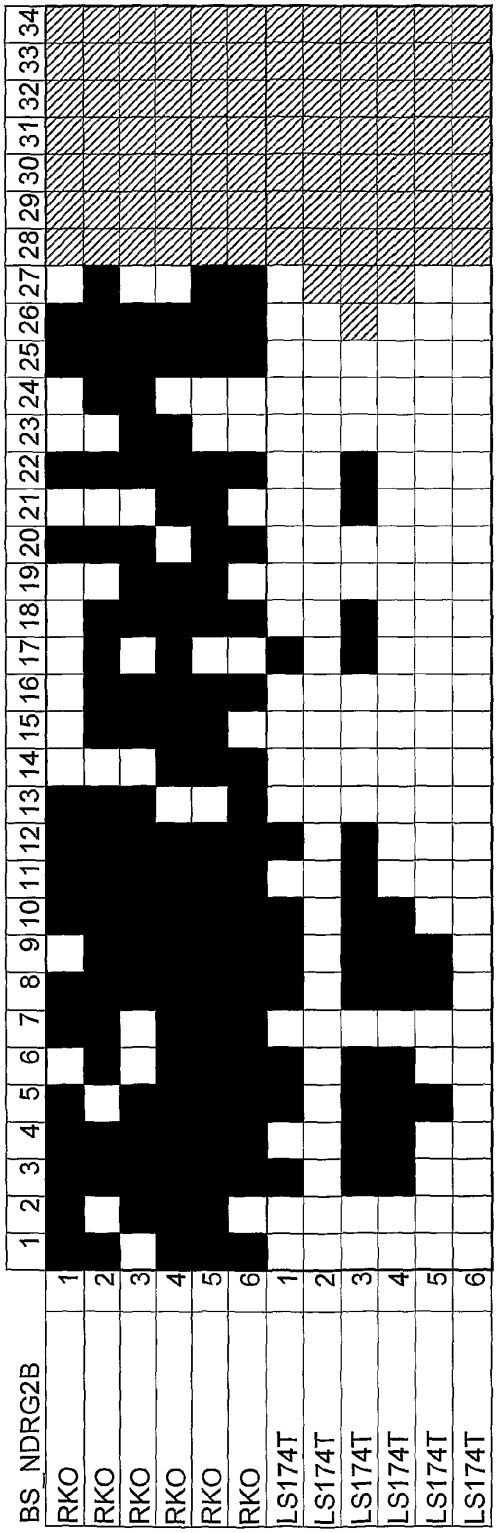


FIG. 1b

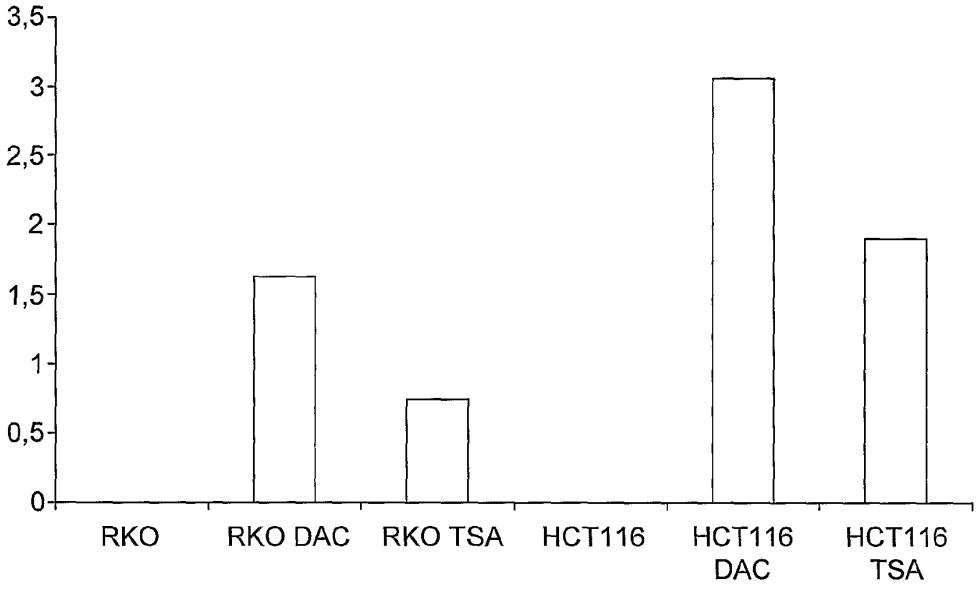

3,5
3
2,5
2
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1
0,5
0
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RKO DAC
RKO TSA
HCT116
HCT116 DAC
HCT116 TSA

FIG. 2

gtttaatagagttttgtatggaaaagattggaatttagggaggagtagagtttCGtttaa
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FIG. 3a

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gaggggggtggtggaatgttttggtgagtCGagtagagtttggaagCGtttagtCGttCGt
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gggggggCGtgtttattgggggttttggggCGggttttgCGgggagtttagCGttCGtgggCG

FIG. 3b

ggttggggCGggtCGggggCGggtttgCGggaagttCGagtCGgggCGggttCGagttaa
aggtaagtgaaggtggaagCGgtCGCGgCGgtagtaggtaggggggaggggtaggCGaggg
gtCGtagggtttggaaggCGttagtCGggtCGgCGggCGgtgtgattgattCGCGttttt
tggagttggaggttCGggggaaagggttagtaCGgagCGggCGttCGgttgttgCGtata
aaggttgaggttttaagagttgtagggCGtgtttggggtgCGCGCGaggttgtgtgtaag
gttCGgggCGtttggtatCGgttaCGtaagggtgttagtttttaCGCGgaCGggttgCGt
agggattCGtagggaCGggtttCGggtgttgggttCGgtCGgtttttttgttttattta
tttttttCGatggCGtttgttttCGCGggtCGtCGggggagaaCGggagaagattgtgg
gatCGtttttttttttttttttttattCGtgggatttataCGgagattgagtagaggaga
gaagCGaCGgtattaattttgtgtttgtttttaaattgtggagggaaatagttatgttt
tgttatttttttttattggagagttgttaaaaattttggggatttttattttttCGCG
tttagttttttgtagatagaaaattgaggttaaatgtagtaattaattggggtaaggtt
atattgggagtggtagagtCGggattCGaattCGgaggttttgatagggttatttttta
tttttatattagtatttttttttttttttCGCGtagtttgaattgatgttttgtagttt
atattgatttCGgttCGagtttagagaggaCGtgtttttggatttaggggtagatagat
attggggagggttagtgattCGaggaggttgatgattttgggagtttgggttttggttga
ggtttttCGtttttgaaagattttaggtttgttttttatgttatttatttCGttgttgtg
ttttttagatttagagttagaaggagtgagaattttgattttaatttttattgtattt
agttaataggagtttagtaagtgattttatttCGtaggttgtaggttttttttgtgtag
gtttgtttaggtgttttgttttttatttattaatgttgaatttgCGgagtttatttagag
ttgggaggaagggttttgggaggaagggaggtattgggttggttggatagatgttttttt
tatgttgttttttttattttttttattggttatgttgttttggttatttttagttttttt
tgagttgtCGatttttggggttttaattgaaggttatggttaattgtgaatttttatagtt
tCGttttttggtattttgtttttttagaatatttagttattgagttataagttttatttttt
tatttgagagtgtggggtggttttttaaatatagttCGagaaatttgagttatatttttgt
tttattttggtattttggtttagggtagaggagggaggggtttggaggtgtggtgttgag
ggtggtagtggggatggtgagtattaagagaagggttgttttgatttttttttttaaagtt
tttatCGattagtttggaaaCGgagttgggggtaggaaaggtttttttgtgtgttttagag
tagttttaatttttgggttttttttgttttttgtatttttttgtgttgagagaggaagtt
tggtgtttgaggCGatgtggggatgtagagataatttttttagtttttatttttttatttg
ttaggttattatggCGgagttgtaggaggtgtagattatagaggagaagttattgttgtt
aggatagaCGtttgaggCGgttaaggttattagagatCGtttgatttttatgtttaaatt
ttagattttttaagtatttttttttgttttttaatttttttaagCGtattgattttatttaa
tttatattattgtttattattattttagtttttttttttta

FIG. 3b

CONT'D

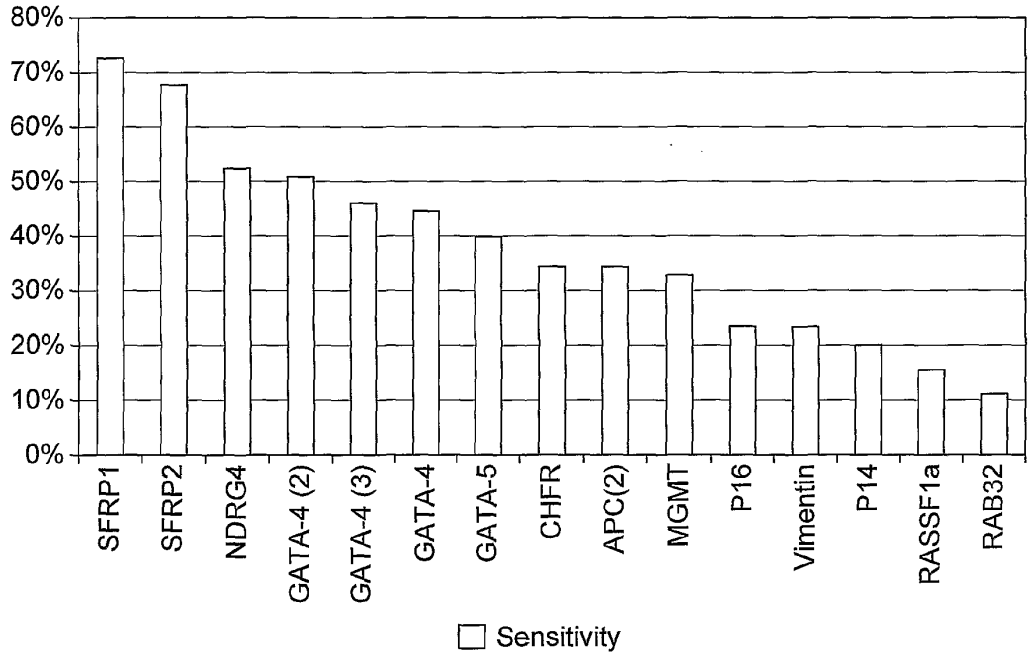

80%
70%
60%
50%
40%
30%
20%
10%
0%
SFRP1
SFRP2
NDRG4
GATA-4 (2)
GATA-4 (3)
GATA-4
GATA-5
CHFR
APC(2)
MGMT
P16
Vimentin
P14
RASSF1a
RAB32
Sensitivity

FIG. 4

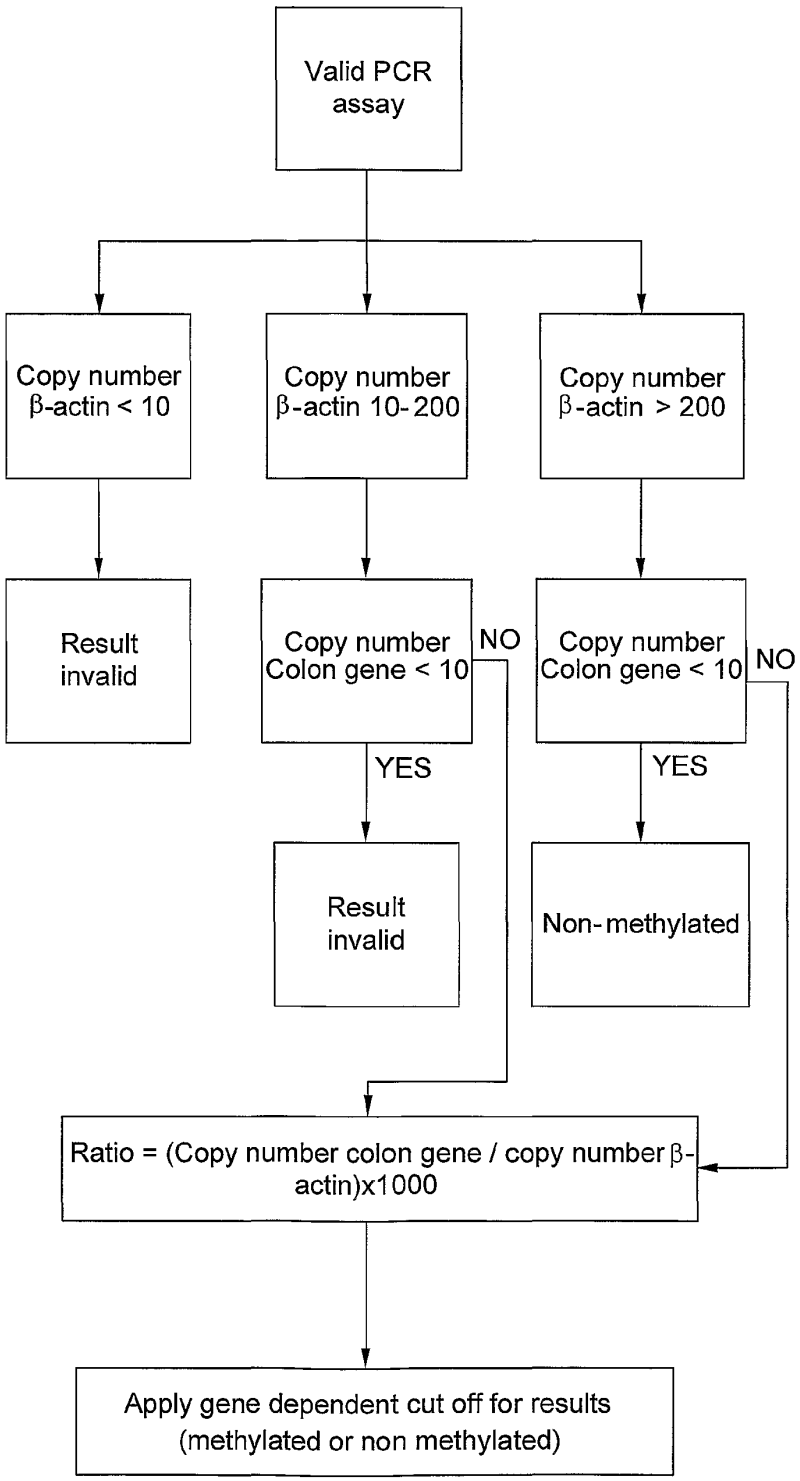

Valid PCR assay
Copy number β-actin < 10
Copy number β-actin 10-200
Copy number β-actin > 200
Result invalid
Copy number Colon gene < 10
NO
Copy number Colon gene < 10
NO
YES
YES
Result invalid
Non-methylated
Ratio = (Copy number colon gene / copy number β-actin)x1000
Apply gene dependent cut off for results (methylated or non methylated)

FIG. 5

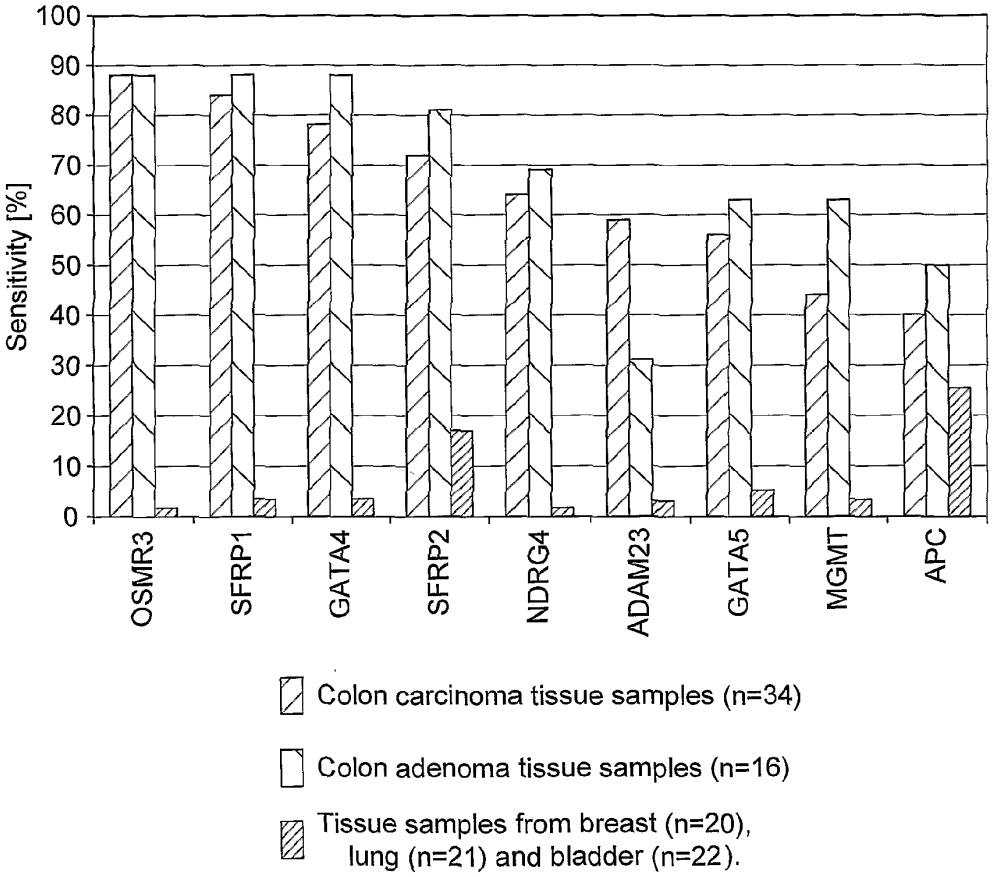

100
90
80
70
60
50
40
30
20
10
0
Sensitivity [%]
OSMR3
SFRP1
GATA4
SFRP2
NDRG4
ADAM23
GATA5
MGMT
APC
Colon carcinoma tissue samples (n=34)
Colon adenoma tissue samples (n=16)
Tissue samples from breast (n=20), lung (n=21) and bladder (n=22).

FIG. 6

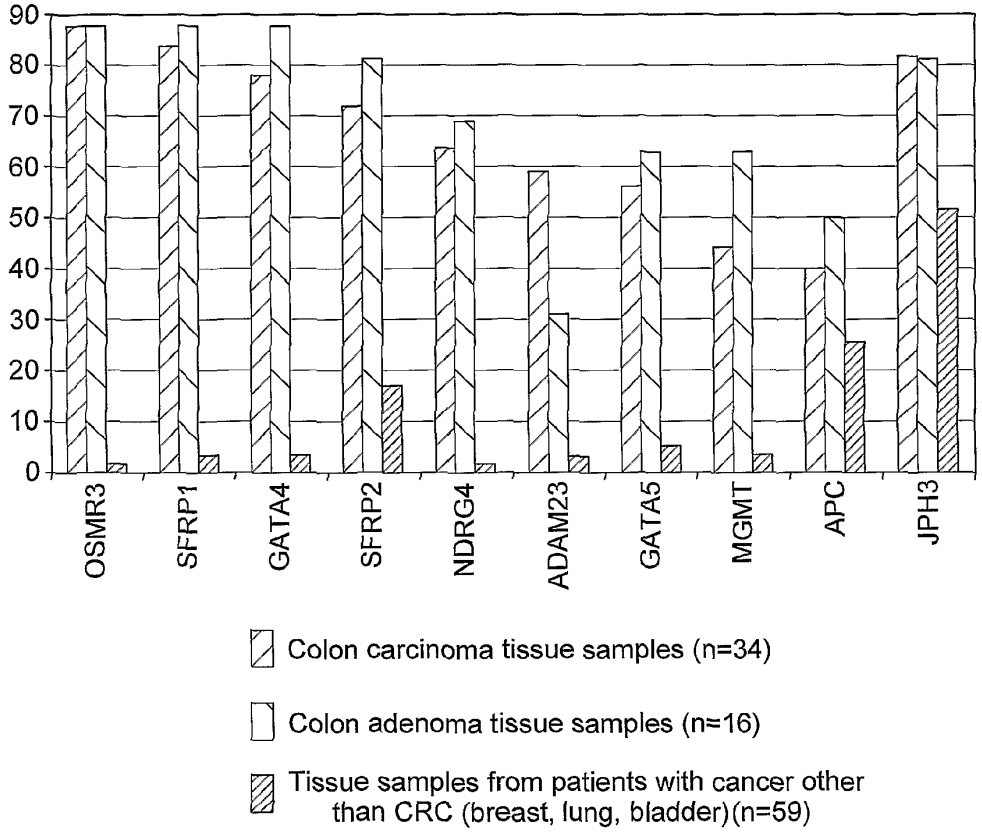

90
80
70
60
50
40
30
20
10
0
OSMR3
SFRP1
GATA4
SFRP2
NDRG4
ADAM23
GATA5
MGMT
APC
JPH3
Colon carcinoma tissue samples (n=34)
Colon adenoma tissue samples (n=16)
Tissue samples from patients with cancer other than CRC (breast, lung, bladder)(n=59)

FIG. 7

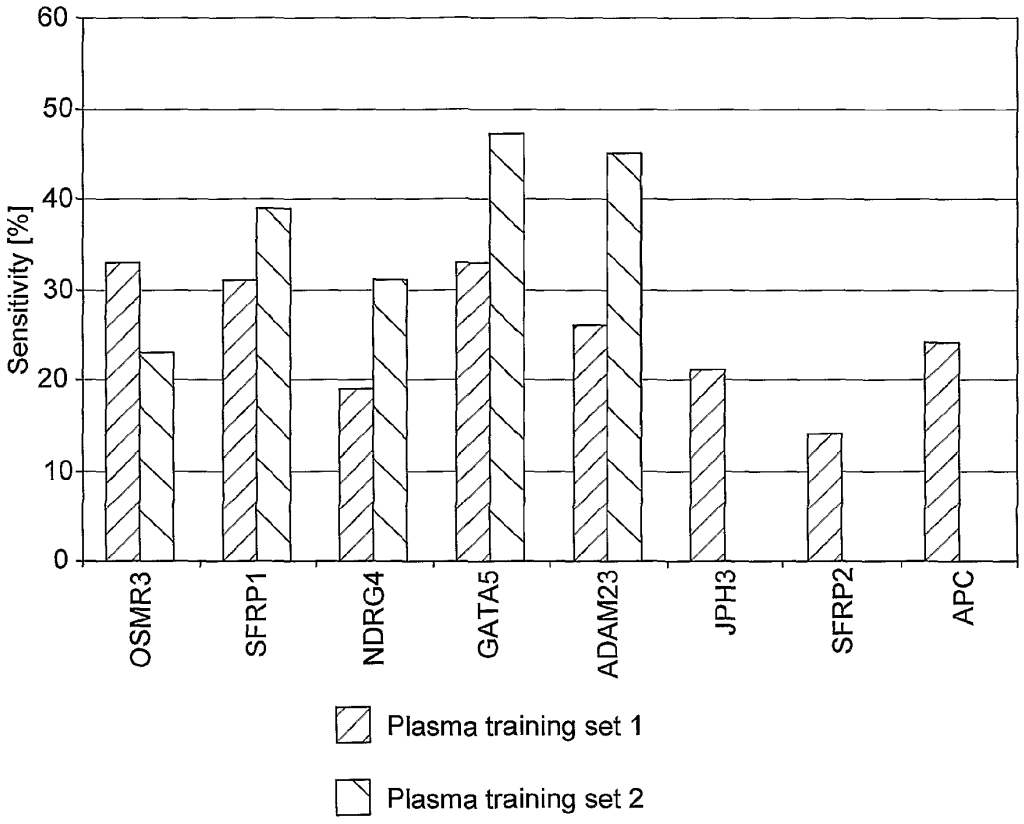

60
50
40
30
20
10
0
Sensitivity [%]
OSMR3
SFRP1
NDRG4
GATA5
ADAM23
JPH3
SFRP2
APC
Plasma training set 1
Plasma training set 2

FIG. 8

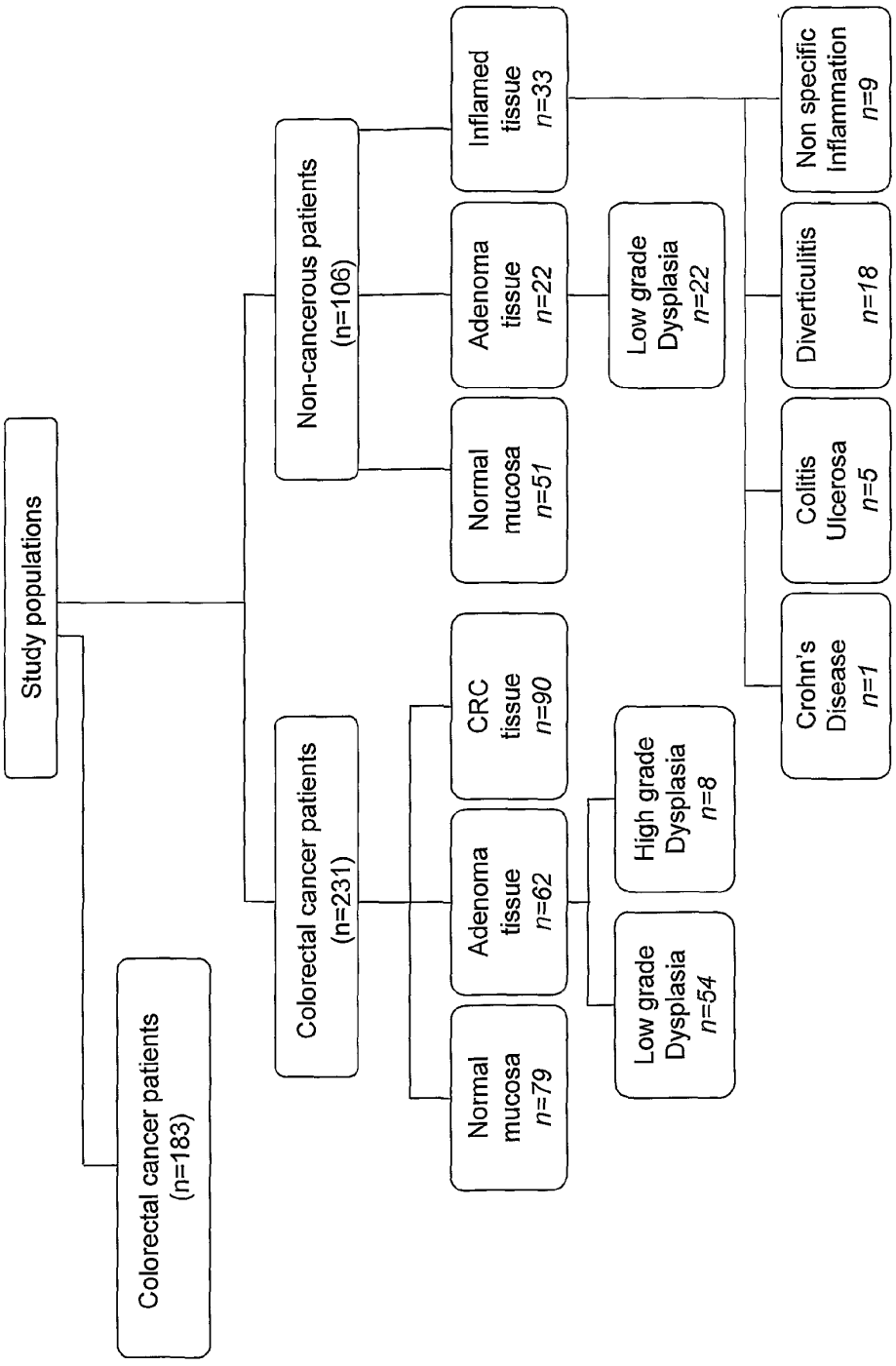


FIG. 9

Geneoscopy Exhibit 1046, Page 270

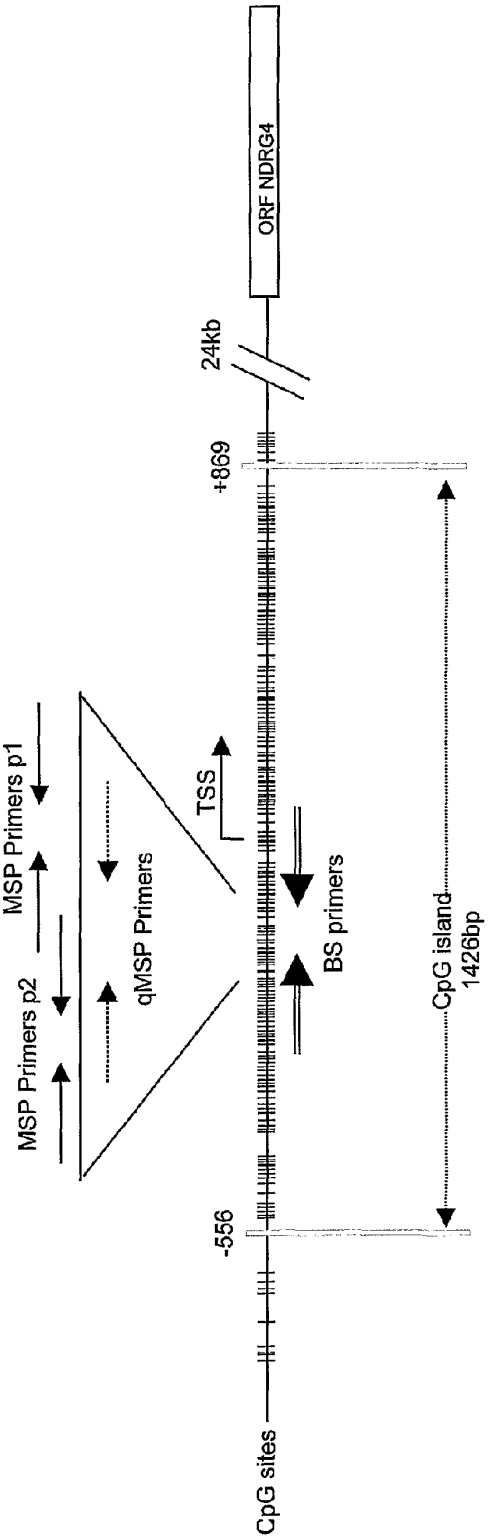

ORF NDRG4
24kb
+869
-556
CpG sites
MSP Primers p1
MSP Primers p2
qMSP Primers
TSS
BS primers
CpG island
1426bp

FIG. 10

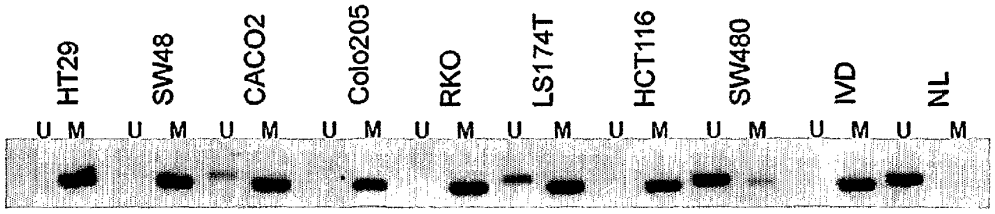


FIG. 11a

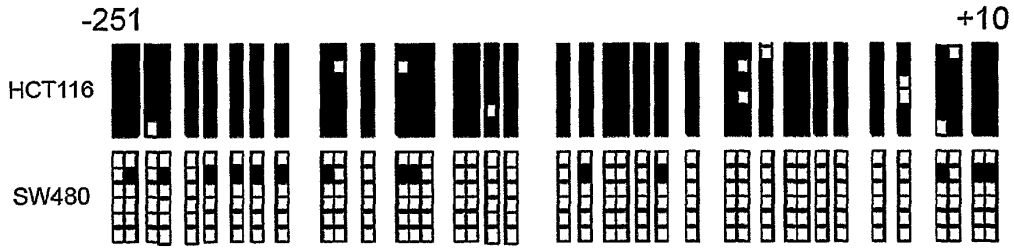


FIG. 11b

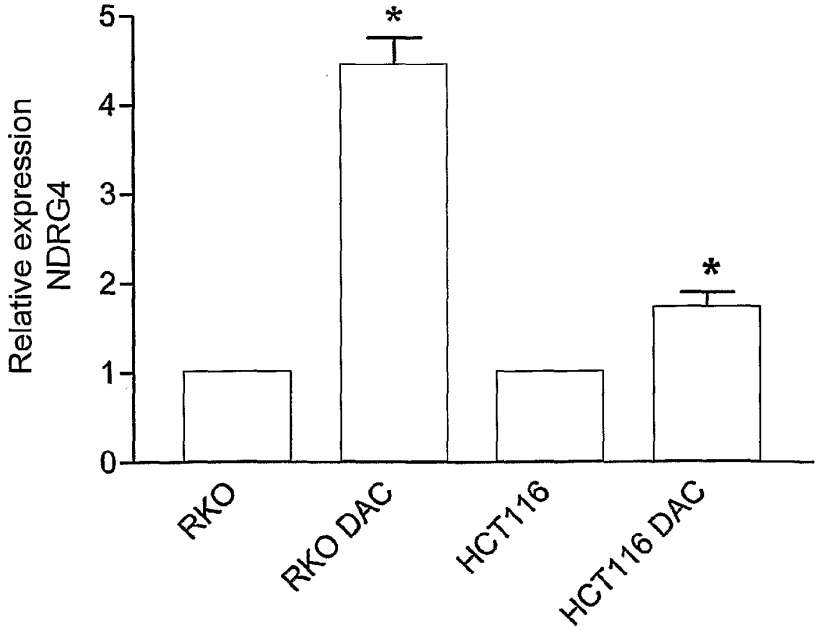


FIG. 11c

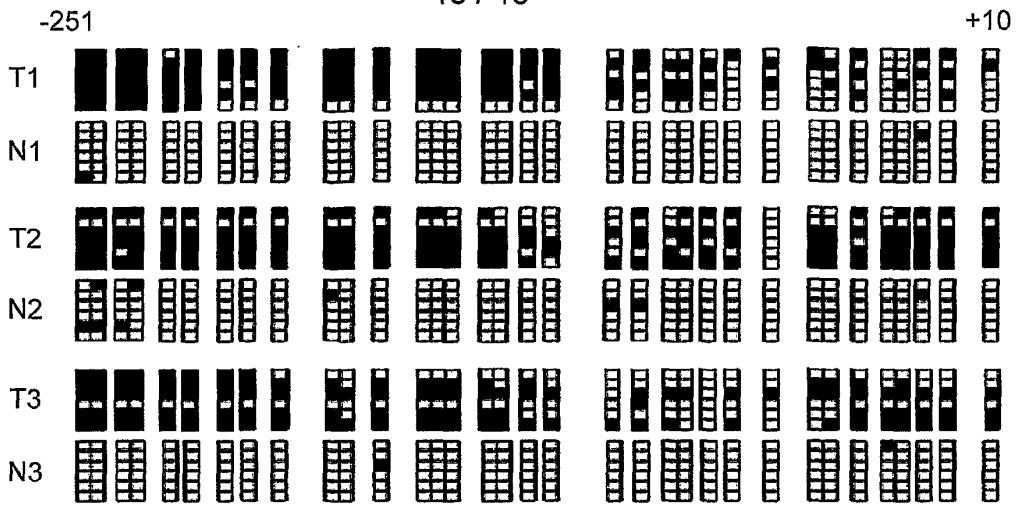


FIG. 12a

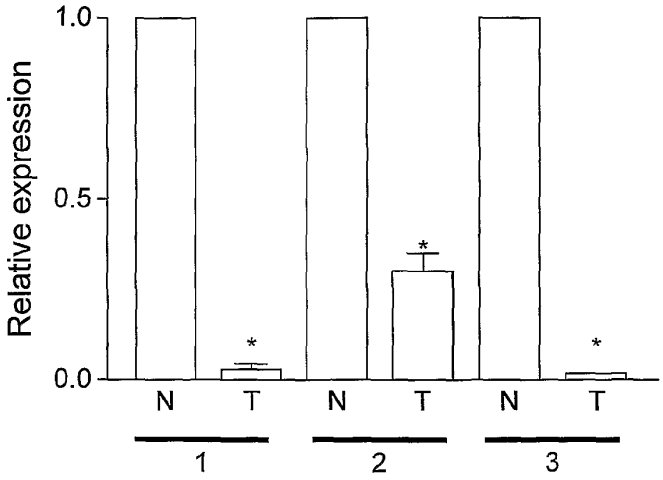


FIG. 12b

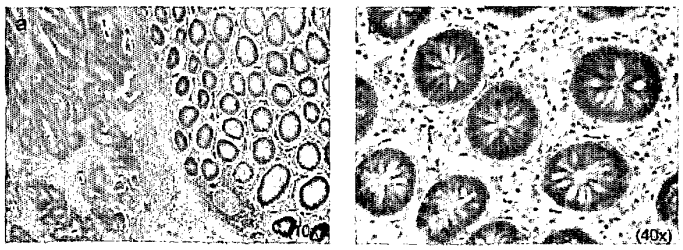


FIG. 12c

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/000056

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/113770 A (EPIGENOMICS INC [US]; BALLHAUSE MATTHIAS [DE]; BERLIN KURT [DE]; DEVOS) 26 October 2006 (2006-10-26) abstract page 9, line 15 - line 35; figure 1 page 14, line 16 - page 15, line 11 page 19, line 13 - line 20 page 21, line 10 - page 24, line 15 page 67, line 25 - page 74, line 12 page 95, line 10 - page 101, line 14 claims 6,53-62,64 -/--	99-134

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

[X] See patent family annex.

* Special categories of cited documents :

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

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2008	20/06/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tilkorn, A

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHOU R-H ET AL: "Characterization of the Human NDRG Gene Family: A Newly Identified Member, NDRG4, Is Specifically Expressed in Brain and Heart" GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 73, no. 1, 1 April 2001 (2001-04-01), pages 86-97, XP004432256 ISSN: 0888-7543 abstract page 87, column 2, paragraph 2 - paragraph 5; figure 26 page 88, column 1, paragraph 1 page 94, column 2, paragraph 2 - page 95, column 1, paragraph 2	171-173
A	QU XIANGHU ET AL: "Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family" MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 229, no. 1-2, January 2002 (2002-01), pages 35-44, XP002479836 ISSN: 0300-8177 abstract page 36, column 1, paragraph 3 - column 2, paragraph 1 page 38, column 1, paragraph 1 page 41, column 1, paragraph 2 - column 2, paragraph 2 page 43, column 2, paragraph 2	1-7, 9-25, 27-33, 35-62, 65-70, 72-77, 79-84, 86-94, 96-124, 126-134, 153-163, 170-173
A	LUSIS ERIKS A ET AL: "Integrative genomic analysis identifies NDRG2 as a candidate tumor suppressor gene frequently inactivated in clinically aggressive meningioma." CANCER RESEARCH 15 AUG 2005, vol. 65, no. 16, 15 August 2005 (2005-08-15), pages 7121-7126, XP002479837 ISSN: 0008-5472 cited in the application abstract page 7122, column 2, paragraph 2 page 7124, column 2, paragraph 2 - page 7126, column 2, paragraph 1 page 7124, column 2, paragraph 2 - page 7126, column 2, paragraph 1 page 7122, column 2, paragraph 2 - page 7123, column 1, paragraph 1	1-7, 9-25, 27-33, 35-62, 65-70, 72-77, 79-84, 86-94, 96-124, 126-134, 153-163, 170-173

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VERMA M ET AL: "Epigenetics in cancer: implications for early detection and prevention" LANCET ONCOLOGY, LANCET PUBLISHING GROUP, LONDON, GB, vol. 3, no. 12, 1 December 2002 (2002-12-01), pages 755-763, XP004811872 ISSN: 1470-2045 abstract page 757, column 2, paragraph 4 - page 759, column 1, paragraph 2 page 761, column 1, paragraph 3 - column 2, paragraph 3; figure 2; table 1	1-7, 9-25, 27-33, 35-62, 65-70, 72-77, 79-84, 86-94, 96-124, 126-134, 153-163, 170-173

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2008/000056

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 61-64, 95 (completely),
1-7,9-25,27-33,35-60,65-70,72-77,79-84,
86-94,96-124,126-135,153-163
, 170-173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in NDRG4

---

2. claims:
1,3-6,9-14,18,19,27-29,31-33,35-54,56,
57,60,65,67-69,72,75,76,79,81
-83,86-94,96-98,102,103,108-111,153-163,
170-173

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in NDRG2

---

3. claims:
1-3,8-13,16,18-23,26-33,35-48,50-56,59,
65-67,70-74,78-81,85-87,89-9
3,96-106,108-111,125-130,133-135,
153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in GATA4

---

4. claims:
1-3,7-13,16-33,35-48,50-56,58,59,65-67,
70-74,77-81,84-87,89-93,96-1
30,133-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in OSMR

---

5. claims:
1-3,7-13,17-20,23,25-33,35-48,50-56,58,
59,65-67,70-74,77-81,84--87,
89-93,96-100,102,103,107-124,
126-130,133-135,153-163,170-173

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in GATA5

---

6. claims:
1-3,7,9-13,18-21,23,27-33,35-48,50-56, 58,65-67,70,72-74,77,79-81,84 ,86,87,89-93,96-100,102,107-114,116,117, 119-124,126-135,153-163,170 -173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in SFRP1

---

7. claims:
1-3,7-13,17-19,23-33,35-48,50-59,65-67, 70-74,77-81,84-87,89-93,96-1 00,102,103,107-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in ADAM23

---

8. claims:
1-3,7,9-13,18,27-48,50-58,65-67,70, 72-74,77,79,81,84,86,87,89-93,96 -100,102,108-114,116,117,119-124, 126-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in JPH3

---

9. claims:
1-3,7,9-13,16,18-22,27-33,35-48,50-58, 65-67,70,72-74,77,79-81,84,86 ,87,89-93,96-106,108-114,116,117, 119-124,133-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in SFRP2

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

10. claims:
1-3,7,9-13,18-21,27-33,34-48,50-58,
65-67,70,72-74,77,79-81,,84,86,8
7,89-93,96-100,102-103,108-114,116,
119-124,153-163,170-173 (in
part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in APC

---

11. claims:
1-3,9-13,18-20,23,27-33,35-48,50-56,
65-67,72-7479-81,86,87,89-93,96
-100,102.103,108-111,
126-135,153-163,170-173 (in part)

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in MGMT

---

12. claims:
1,3,9-13,18,19,27-48,50-54,56,58,65,
67,72,74,77,79,81,84,86,89-93,9
6-99,102,103,108-112,114,116,117,
119-124,126-135,153-163,170-173
(in part

methods, kits primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in TFPI2

---

13. claims:
1,3,9-13,18,19,27-29,31-48,50-54,56,
58,65,67,70,72,74,77,79,81,84,8
6,87,89-93,96-98,102,103,108-112,114,
116,117,119-124,126-135,153-16
3,170-173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in BNIP3

---

14. claims:

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in FOXE1

---

15. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in SYNE1

---

16. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in SOX17

---

17. claims:

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in PHACTR3

---

18. claims:

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1,3,9-13,18,19,27-48,50-54,56,58,65,
67,70,72,74,77,79,81,84,86,87,8
9-93,96-98,102,103,108-112,114,116,
117,119-124,126-135,153-163,170-
173 (in part)

methods, kits, primers and probes for detecting a predisposition to or the incidence of cancer in a sample, method of treatment and its likelihood of success on the basis of an epigenetic change in JAM3

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19. claims: 136-152 (completely), claims 153-157 (in part)

Method of determining the methylation status in a blood sample

---

20. claims: claims 164-170

Method of processing a faecal sample to isolate and prepare DNA

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2008/000056

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006113770 A	26-10-2006	CA 2603815 A1	26-10-2006
		EP 1871912 A1	02-01-2008

US011634781C1

(12) **EX PARTE REEXAMINATION CERTIFICATE** (12448th)

United States Patent
Louwagie

(10) **Number: US 11,634,781 C1**
(45) **Certificate Issued: Dec. 4, 2023**

(54) **FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**

(71) Applicant: **Exact Sciences Corporation**, Madison, WI (US)

(72) Inventor: **Joost Louwagie**, Domach (CH)

(73) Assignee: **EXACT SCIENCES CORPORATION**, Madison, WI (US)

Reexamination Request:
No. 90/015,237, May 22, 2023

Reexamination Certificate for:
Patent No.: **11,634,781**
Issued: **Apr. 25, 2023**
Appl. No.: **17/936,335**
Filed: **Sep. 28, 2022**

Related U.S. Application Data

(63) Continuation of application No. 15/634,607, filed on Jun. 27, 2017, which is a continuation of application No. 15/010,436, filed on Jan. 29, 2016, now abandoned, which is a continuation of application No. 13/147,570, filed on Mar. 12, 2012, now abandoned.

(51) **Int. Cl.**
C12Q 1/6886 (2018.01)
G01N 33/574 (2006.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/6886*** (2013.01); ***G01N 33/57446*** (2013.01); *C12Q 2600/106* (2013.01); *C12Q 2600/154* (2013.01); *C12Q 2600/158* (2013.01); *G01N 2333/805* (2013.01); *G01N 2800/50* (2013.01); *G01N 2800/52* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

To view the complete listing of prior art documents cited during the proceeding for Reexamination Control Number 90/015,237, please refer to the USPTO's Patent Electronic System.

Primary Examiner — Krisanne M Jastrzab

(57) **ABSTRACT**

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

EX PARTE REEXAMINATION CERTIFICATE

NO AMENDMENTS HAVE BEEN MADE TO THE PATENT

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

The patentability of claims **1-20** is confirmed.

* * * * *

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

U.S. PATENTS					
Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		5198365	1993-03-30	GROW et al.	
		5741650	1998-04-21	LAPIDUS et al.	
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		9891223	2019-02-13	BEAULIEU et al.	
		11634781	2023-04-25	LOUWAGIE	

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Examiner Initials*	Cite No.[3]	Document Number Number-Kind Code[4] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20060216714	2006-09-28	KANAOKA	
		20120164238	2012-06-28	JOOST	
		20160194723	2016-07-07	LOUWAGIE	

Note: Submission of copies of U.S. Patents and published U.S. Patent Applications is not required.

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Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[5] Number[6] Kind Code[7] *(if known)*	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translation[8]
		WO 1997/25925	1997-07-24	HULIOT PLASTICS INDUSTRIES et al.		
		WO 2005/113769	2005-12-01	EXACT SCIENCES CORPORATION		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		

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Sheet	2	of	7	*Attorney Docket Number*	EXCTD-35239.307

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Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and-or country where published.	Translation[6]
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		USPTO Final Office Action for US Patent Publication No. US15/010,436 dated October 28, 2016, 15 pages	
		Recorded Assignment of US Patent No. 11,634,781 dated April 25, 2017, 5 pages	
		USPTO Nonfinal Office Action for US Patent Publication No. US17/936,335 dated January 11, 2023, 7 pages	
		Request for Ex Parte Reexamination of U.S. Patent 11,634,781, filed May 22, 2023, 286 pages	

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				Art Unit	1634
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		USPTO Order Granting Request for Ex Parte Reexamination of US Patent No. 11,634,781 dated June 29, 2023, 18 pages	
		USPTO Notice of Intent to Issue Ex Parte Reexamination Certificate for US Patent No. 11,634,781 dated Oct 18, 2023, 5 pages	
		Ex Parte Reexamination Certificate for US Patent 11,637,781, issued December 4, 2023, 2 pages	
		Petition for *Inter Parties* Review of U.S. Patent 11,634,781, filed January 11, 2024, 87 pages	

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				Examiner Name	WHISENANT, Ethan C.
Sheet	8	of	7	*Attorney Docket Number*	EXCTD-35239.307

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

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

SIGNATURE

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

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

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(30) Priority Data:
116824 19 January 1996 (19.01.96) IL

(71) Applicants *(for all designated States except US)*: HULIOT PLASTICS INDUSTRIES [IL/IL]; Kibbutz Sdeh Nehemia, 12145 Mobile P.O. Upper Galilee (IL). QUALITY 9000 LTD. [IL/IL]; P.O. Box 593, 76103 Rehovot (IL).

(72) Inventor; and
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(74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel-Aviv (IL).

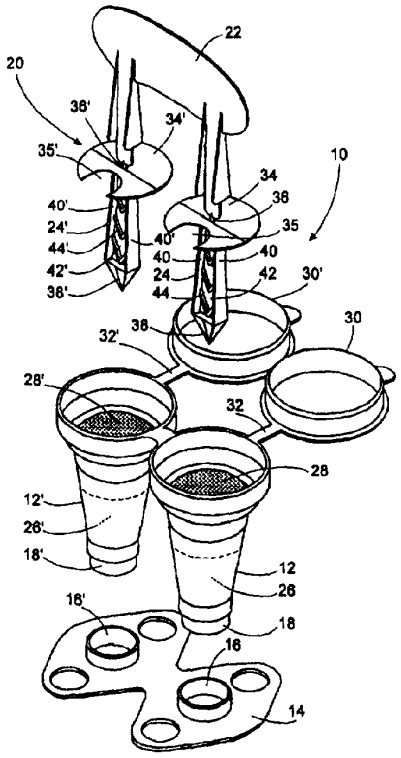
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: COLLECTION AND TRANSPORTATION OF A PASTY MATERIAL SAMPLE

(57) Abstract

A system for collecting, storing and transporting of specimens of a pasty material, e.g. stool is provided. The system comprises at least one container holding a preservation medium for preserving a chemical entity or living matter to be assayed in a specimen, the container being sealed by a pierceable membrane having a closure arrangement for sealing the container after the membrane is pierced. The system further comprises a sampling device having a handle and at least one elongated specimen collecting member detachably attached thereto, the collecting member being adapted to be received within the container and having the pasty material retaining portion and a piercing tip for piercing the membrane at a lowermost end thereof remote from said handle, for piercing said film. Also provided is a stool collecting device for easy collecting of excreted stool.



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COLLECTION AND TRANSPORTATION OF A PASTY MATERIAL SAMPLE

FIELD OF THE INVENTION

The present invention concerns a system for the collection and transportation of a pasty material sample, for example of stool, which is to be distributed in a transport and/or suspension medium.

BACKGROUND OF THE INVENTION

Collection and transportation of a pasty material, such a stool, food samples, soil samples and the like for the purpose of laboratory manipulation such as detection of microorganism being bacteria or parasites, detection of cryptic blood, preparation of a biochemical profile, etc., is an essential component of modern medicine, health care and environmental management.

Often, when the pasty material is transported to the laboratory without a preservation medium, by the time it reaches a laboratory no viable micro-organisms can be detected, and the result of the laboratory tests are thus distorted.

In order to overcome this problem, various devices and systems for the collection and transportation of pasty material have been developed, in which the pasty material is distributed in a medium capable of maintaining viable microorganisms. Usually, such devices comprise a container holding the medium, and a sampler capable of picking up a small portion of the pasty material and placing it within said container.

US 5,149,506 discloses a device for collection and transportation of a stool sample which is composed of a container with a lid having a spoon secured to the underside of the lid, the container being filled with a liquid capable of maintaining microorganisms in a viable form.

US 4,559,839 discloses a stool collection and concentration receiver comprising a container holding a medium and including within it a collecting-filtering device comprising a stick for picking up the stool sample and sieve through which the diluted stool is filtered.

US 4,678,559 discloses a sampling vessel for pasty sample material comprising a sample-taking cup fitted on the lid of a container and facing towards the container's bottom.

In the above three patents the sampling member, used to pick the sample of the pasty material, is held *a priori* within the container holding also the preservation medium. This feature has the following problems:

- in order to sample the pasty material the container holding both the medium and the sampling member has to be opened, thus increasing the chances of contamination of the sterile medium by ambient atmosphere, or by the hands of the user and increases the possibility of spillage due to mishandling;
- once the sampling member is taken out of the medium filled container it may drip the medium on to its surrounding, and where the medium contains harmful ingredients, e.g. formaldehyde or mercury which are present in some preservation media, this may cause some damage if it comes into contact with the user's skin or eyes;
- the wet sampling member, once coming into contact with the pasty material sample, adds to the sample also a certain amount of the transport or suspension medium itself. This may be problematic if another sample of the pasty material is to be transferred into another container holding a different medium, since the second sample may already be contaminated by the first medium.

A stool sample is typically subjected to two different tests one being a bacteriological test and the other a parasitological test. The medium

required to maintain viable bacteria and the medium required to maintain viable parasites are not only different but are mutually exclusive, i.e. one type of medium damages the other type of microorganism. Thus, in the case of a pasty material which is to be sampled for the purpose of two or more different laboratory assay, such as stool, it would be preferable to provide a system for collection and transportation of pasty material which comprises two or more separate containers each one holding a different transport or suspension medium.

Finally, the above-mentioned prior art devices for collection and transportation of stool, do not address the problem of obtaining the original stool sample in a convenient manner which enables easy access to the user and the prior art tends to leave the solution to the problem to the skills of each user.

The present invention intends to provide an answer to some of the above-noted problems.

GENERAL DESCRIPTION OF THE INVENTION

The present invention provides a system for collecting, storing and transporting of specimens of a pasty material, comprising:

at least one container holding a preservation medium for preserving a chemical entity or living matter to be assayed in the specimen, the container being sealed by a pierceable membrane and having a closure arrangement for sealing the container after the membrane is pierced; and

a sampling device having a handle and at least one elongated specimen collecting member detachably attached thereto, the collecting member being adapted to be received within the container and having a pasty material retaining portion and a piercing tip for piercing the membrane at a lowermost end thereof remote from said handle, for piercing said film.

The system of the present invention is suitable for collecting, storing and transporting of any pasty material, for example, stools, food samples, e.g. soft cheese, yogurt, various spreads; soil samples, e.g. mud samples; and many others. With appropriate preservation media, the

samples may be stored for a lengthy period of time prior to assaying for the chemical entity or living matter. The composition of the medium depends on the type of entity which should be preserved and subsequently assayed: for example, if the entity is a chemical substance, the medium will have a composition which ensure that the substance is preserved in a manner to avoid its deterioration; if the assayed entity is microorganism or any other living matter, the medium will be designed so as to maintain such microorganisms or other living matter in a viable condition. A system for collecting, storing and transporting specimens to subsequent assays for living matter, e.g. microorganisms, yeasts or parasites thereon, is a preferred embodiment of the invention. In accordance with a particularly preferred embodiment, the pasty material is stool.

At times a variety of different entities have to be assayed in the pasty material. This is the case, for example, in stool, where it is generally desired to assay both bacteria and parasites, which as noted above, require different media. A system for assaying a plurality of entities, which require different media, comprises at least two containers, each holding a different preservation medium, each suitable for different entities. The containers in such a system may be permanently attached to one another; or alternatively, the attachment of the two containers to one another being in a detachable fashion to allow detachment when the containers with the sample reaches the laboratory.

The closure arrangement is typically a rigid lid member attached to the container through a flexible linker.

The system further comprises a sampling device which is an element distinct and separate from the container. The provision of the container and a separate sampling device ensures that until the pasty material sample is introduced into the container, the medium therein is kept totally sterile. As already noted above, in prior art devices, where a sampling device was stored within the container, it was necessary to open the container prior to sampling, with an inevitable loss of sterility.

The sampling device comprises a handle which is detachably connected to the at least one collecting member. The detachable connection can be by a breakable portion, by a fastener capable of holding or releasing an attachment element in the collecting member, etc. The at least one collecting member is adapted to be received within corresponding at least one container. (It will be appreciated, that the number of collecting members will typically correspond to the number of containers, and where the containers are held together, the spacing between the collecting members will be made to correspond to the distance between the container such that all collecting members may be inserted simultaneously to the respective containers).

Each collecting member has a piercing tip for piercing the container's pierceable membrane and has a pasty material retaining portion. Once the membrane is pierced by the piercing tip, the collecting member is pushed down until the material retaining portion is accommodated within the container and embedded in the medium. Then, the handle is detached from the collecting member. As will be appreciated, there are a number of different constructions which have the capability of retaining a specimen of the pasty material: for example, a rough surface having multiple depressions and/or protrusions; a helical structure; a ladder-like shaped structure having straight, curved or V-shaped ribs; a structure comprising a planar stem with protrusions, which may have the form of straight, curved or V-shaped ribs, or which may comprise a plurality of bulges, etc.

In order to ensure that the pasty material will be retained on the retaining portion and will not be smeared on the membrane, it is preferable that the pasty material retaining portion has the widest cross-section at the lowermost part, near the piercing tip. This will ensure that the hole in the membrane which will be made by the piercing tip, will be sufficiently large to ensure free passage of the remaining portions of the collecting member. In accordance with an embodiment of the invention, the pasty material retaining portion is defined by two lateral side walls which are wider than the pasty material retaining portion. Thus during insertion, only these side

walls come into contact with the membrane, whereby the majority of the specimen of the pasty material does not come into contact with the membrane.

In accordance with a preferred embodiment of the invention, the sampling device comprises an integral disc with its surface being essentially perpendicular to the longitudinal axis of the collecting member. The shape of the disc is such that once the collecting member is introduced into the container, the disc is snugly retained within the inner walls of the container thus stabilizing the position of the collecting member within the container. The disc preferably defines an opening which allows the insertion of a laboratory sampling device, e.g. a pipette, a sampling needle, etc, for withdrawing a portion of the specimen, this without a need to remove the collecting member with the integral disc.

As can be seen, the system of the invention minimize possible contact between a sample and a laboratory technician, which is especially important in cases where the sample may contain hazardous materials or microorganisms.

In accordance with a preferred embodiment of the invention, as already noted above, the pasty material is stool. In accordance with this embodiment, the system may comprise a stool collecting device, which has a collecting member for collecting an excreted stool sample and attachment members for attachment to a toilet seat. The stool collecting device also forms an independent aspect of the invention. The device is typically made of a sheet, e.g. a paper sheet, with the collecting portion and the attachment member being integral, i.e. made of the same sheet. The attachment member may be a sheet portion adapted for wrapping around a toilet seat and are then retained by virtue of being sandwiched between the toilet seat and the upper edges of the toilet bowl. Alternatively, the attachment member may comprise adhesive patches on a bottom face of the attachment member for adherence therethrough to the toilet seat. The adhesive patches are *a priori* protected with the protection being used prior to use and attachment thereof to the toilet seat.

In accordance with an embodiment of the invention, the stool collecting member is a sheet portion suspended between the attachment members. Alternatively, the stool collecting member may have the form of a sack for collecting excreted stool sample. Such a sack may be provided with openings at its bottom to allow a drainage of excreted fluid while retaining the stool.

The stool collecting device is preferably made of a flushable material, e.g. thin paper, and after use may be dropped into the toilet bowl to be flushed thereby.

The invention will now be further illustrated by the following description of a specific embodiment and by the annexed drawings. The illustrated embodiment concerns a system for collecting, storing and transporting stool samples. However, as will no doubt be appreciated by the artisan, this is an example only and the invention pertains also to systems for collecting specimens of pasty material other than a stool.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an exploded view of a system of the invention comprising two containers and a sampling device with two corresponding collecting members;

Fig. 2 shows the system, with the containers being assembled on a base and with the collecting member holding a stool sample;

Fig. 3 shows the system after combining the sampling device with the containers with the collecting member being inserted in the container;

Fig. 4 shows the system after separating between the handle and the collecting members;

Fig. 5 shows the system after closing of the lid, ready for transportation into the laboratory;

Fig. 6 shows a stool collecting device in accordance with the invention;

Figs. 7 and **8** show stool collecting devices in accordance with other embodiments of the invention, attached to a toilet seat.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Reference is first being made to Fig. 1, which is an exploded view showing the various components of the system, generally designated 10. The system 10 comprises two attached containers 12 and 12', a base member 14 with two receptacles 16 and 16' adapted to receive the bottom ends 18 and 18' of containers 12 and 12'; the system further comprises a sampling device 20 having a handle 22 and two collecting members 24 and 24'.

Containers 12 and 12' hold respective media 26 and 26', which may both be the same, but are typically different, e.g. one being a media for preserving bacteria and the other for parasites. Media 26 and 26' are typically semi-solid media, e.g. agar-based, but may also be a lipid, e.g. a culture media. Media 26 and 26' are contained in a portion of the containers sealed by pierceable membranes 28 and 28', the membranes being made of aluminum foil, a laminate of an aluminum foil and a plastic, (e.g. polyethylene) film, etc. Containers 12 and 12' are provided with respective lids 30 and 30' which are linked to the containers by means of linkers 32 and 32'. Sampling device 20 has integral disc members 34 and 34' at the upper end of specimen collecting members 24 and 24'. Disc 34 and 34' have each respective openings 35 and 35', the functions of which will be explained further below. Handle 22 is connected to disc members 34 and 34' by means of breakable neck portions 36 and 36' by small lateral respective movements of the handle and the collecting members connecting necks 36 and 36' break whereby handle 22 and collecting members 24 and 24' are separated.

Collecting members 24 and 24' are elongated and have each a piercing tip 38 and 38' at its bottom end adapted for piercing membranes 28 and 28'. Collecting members 24 and 24' have two lateral walls 40 and 40' which define the stool retaining portion consisting also of planar members 42 and 42' disposed within the lateral walls and carrying a plurality of V-shaped protrusions 44 and 44'. As can be seen, the collecting members

have the largest cross-section at a portion adjacent piercing tips **38** and **38'** thus the hole which is created in the membranes **28** and **28'** allows easy passage therethrough for the remaining portions of the collecting members. Furthermore, lateral walls **40** and **40'** ensure that the stool specimen held on planar members **42** and **42'** is not removed by the membranes **28** and **28'** during passage of collecting members **24** and **24'**.

In order to collect specimens of pasty material, collecting members **24** and **24'** are inserted into a sample of the pasty material, e.g. stool, and thereby specimens are retained on collecting members **24** and **24'**. Sampling device **20** with stool specimens **50** and **50'** retained on collecting members **24** and **24'** is seen in Fig. 2. Also seen in this figure are containers **12** and **12'** assembled on base member **14**.

After collection of the specimens, the sampling device and the containers are combined by first pressing piercing tips **38** and **38'** against membranes **28** and **28'** and then pushing the collecting members until they are accommodated within containers **12** and **12'** with the collecting members being embedded within the medium containing the containers. Fig. 3 shows the sampling device **20** and containers **12** and **12'** immediately after combining the two. As can be seen, the size of disc members **34** and **34'** are such that they are accommodated snugly within the inner walls of the container stabilizing the collecting member in position.

At a next step shown in Fig. 4, the handle **22** is moved naturally thus breaking necks **36** and **36'** leaving the collecting members within containers **12** and **12'**. At a next step shown in Fig. 5, lids **30** and **30'** are used to close containers **12** and **12'** and at this state the containers are shipped to the laboratory. In the laboratory, the lids are opened to a state similar to that shown in Fig. 4, and then a sampling device, e.g. a pipette, a bacteriologic needle, etc., is inserted through openings **35** and **35'**. Thus, sampling of the specimen for laboratory testing does not require removal of the collecting member, as in the case of prior art devices.

A stool collecting device in accordance with an embodiment of the invention is shown in Fig. 6. The device, generally designated **60** is made of a sheet, e.g. paper, particularly thin paper which is flushable in a toilet. The device has a central collecting member, which is a portion of a sheet having the form of a sack, and has two attachment members **64** and **66** which are of a length such that their ends will reach two opposite sides of a toilet seat. The attachment members **64** and **66** have two adhesive patches **68** and **70**, on bottom faces of the device. Adhesive patches **68** and **70** are *a priori* covered by a protective cover (now shown) and prior to use, the cover is removed and then these patches can adhere to the toilet seat. The device is fixed such that stool excreted by a person sitting on the toilet, falls into a sack-like collecting member **62** for later sampling by the sampling device. As can further be seen in Fig. 6, collecting member has at its bottom four openings **72** which are intended to allow a discharge of fluids which may be excreted together with the stool.

Two other embodiments of a stool collecting device are shown in Figs. 7 and 8, fixed to a toilet seat. The stool collecting device **80** shown in Fig. 7, is in principle similar to device **60** shown in Fig. 6 in that it comprises a central sack-like portion **82** and peripheral attachment flaps **84** and **86**. Device **80** is fixed to a toilet seat **88** by means of adhesive patches, similarly as in the case of device **60** in Fig. 6.

Device **90** shown in Fig. 8, comprises a stool collecting portion **92** which is a sheet, e.g. made of paper, suspended between two attachment members **94** and **96** which are two flaps which are wrapped around two opposite sides of the toilet seat **98**, and are fixed by virtue of being clamped between the toilet seat and the underlying toilet bowl (not shown). In addition a device of this embodiment may also comprise adhesive patches at the bottom face members of **94** and **96** to aid in fixing them to the toilet seat.

Having now finalized the description of the specific embodiment, the artisan will no doubt appreciate that the above specific embodiment is

an example only and a myriad of other designs of the container and the sample device as well as the stool collecting device are possible, all being within the scope of the invention as defined in the appended claims.

CLAIMS:

1. A system for collecting, storing and transporting of specimens of a pasty material, comprising:

at least one container holding a preservation medium for preserving a chemical entity or living matter to be assayed in the specimen, the container being sealed by a pierceable membrane and having a closure arrangement for sealing the container after the membrane is pierced; and

a sampling device having a handle and at least one elongated specimen collecting member detachably attached thereto, the collecting member being adapted to be received within the container and having a pasty material retaining portion and a piercing tip for piercing the membrane at a lowermost end thereof remote from said handle, for piercing said film.

2. A system according to Claim 1, wherein the medium is of a kind which maintains living matter in a viable condition.

3. A system according to Claim 2, wherein the pasty material is stool.

4. A system according to Claim 1, comprising two containers, one holding a medium for bacteria and another for holding a medium for parasites.

5. A system according to Claim 4, wherein the two containers are attached to one another.

6. A system according to Claim 4 or 5, wherein the sampling device comprises two specimen collecting members.

7. A system according to any one of the preceding claims, wherein said collecting member has a piercing tip for piercing the containers' pierceable membrane and has a pasty material retaining portion.

8. A system according to Claim 7, wherein the pasty material retaining portion has the widest cross-section at a lowermost part thereof, near the piercing tip.

9. A system according to any one of the preceding claims, wherein the sampling device comprises an integral disc in an upper end of the collecting member, with its surface being essentially perpendicular to the longitudinal axis of the collecting member.

10. A system according to Claim 9, wherein the shape of the disc is such that once the collecting member is introduced into the container, the disc is snugly retained within the inner walls of the container.

11. A system according to Claim 10, wherein the disc defines an opening which once the collecting member is placed within the container, allows insertion of a laboratory sampling device into the medium, without a need to remove the collecting member.

12. A stool collecting device which has a collecting member for collecting an excreted stool sample and attachment members for attachment to a toilet seat.

13. A device according to Claim 11, made of a sheet with a stool collecting portion and integral flats for attachment to a toilet seat.

14. A device according to Claim 12, comprising adhesive patches for fixing the device into a toilet seat.

15. A system according to any one of Claims 1-10, comprising a stool collecting device according to any one of Claims 11-13.

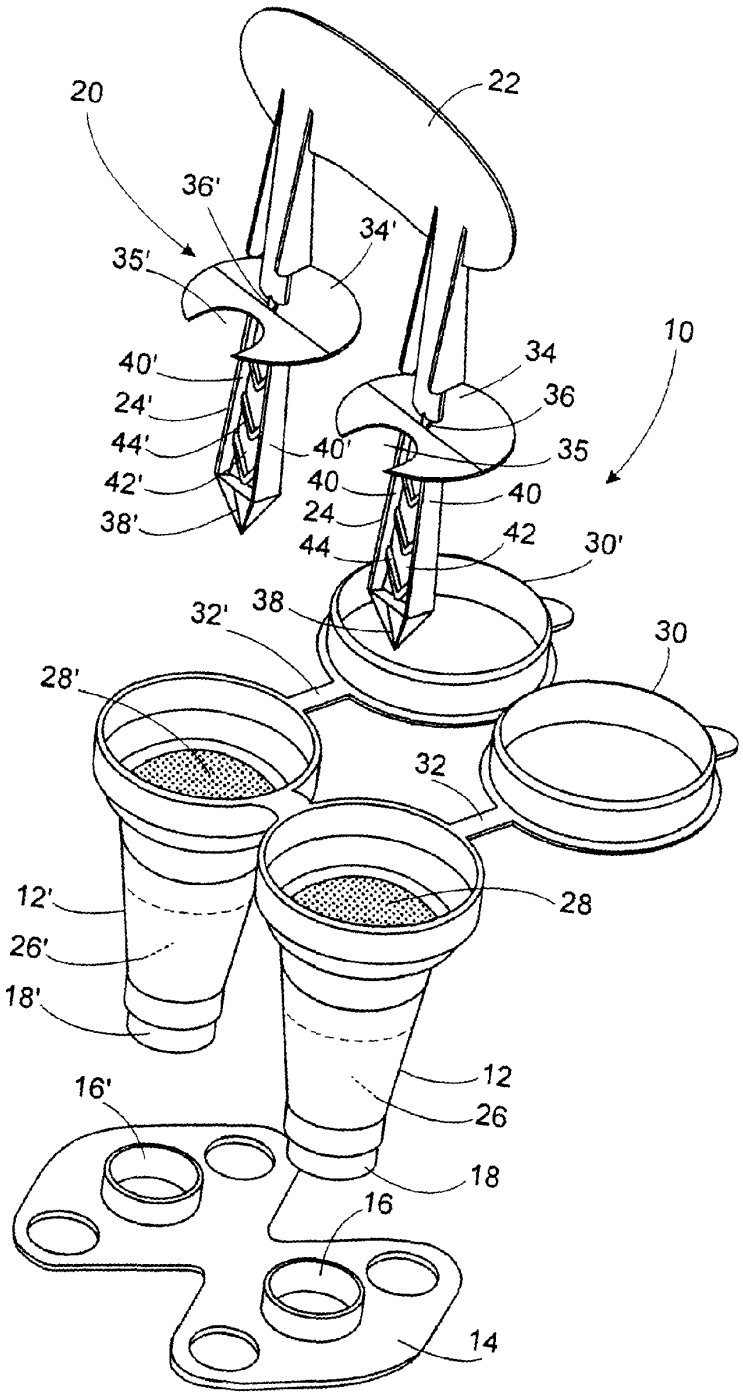

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Fig. 1

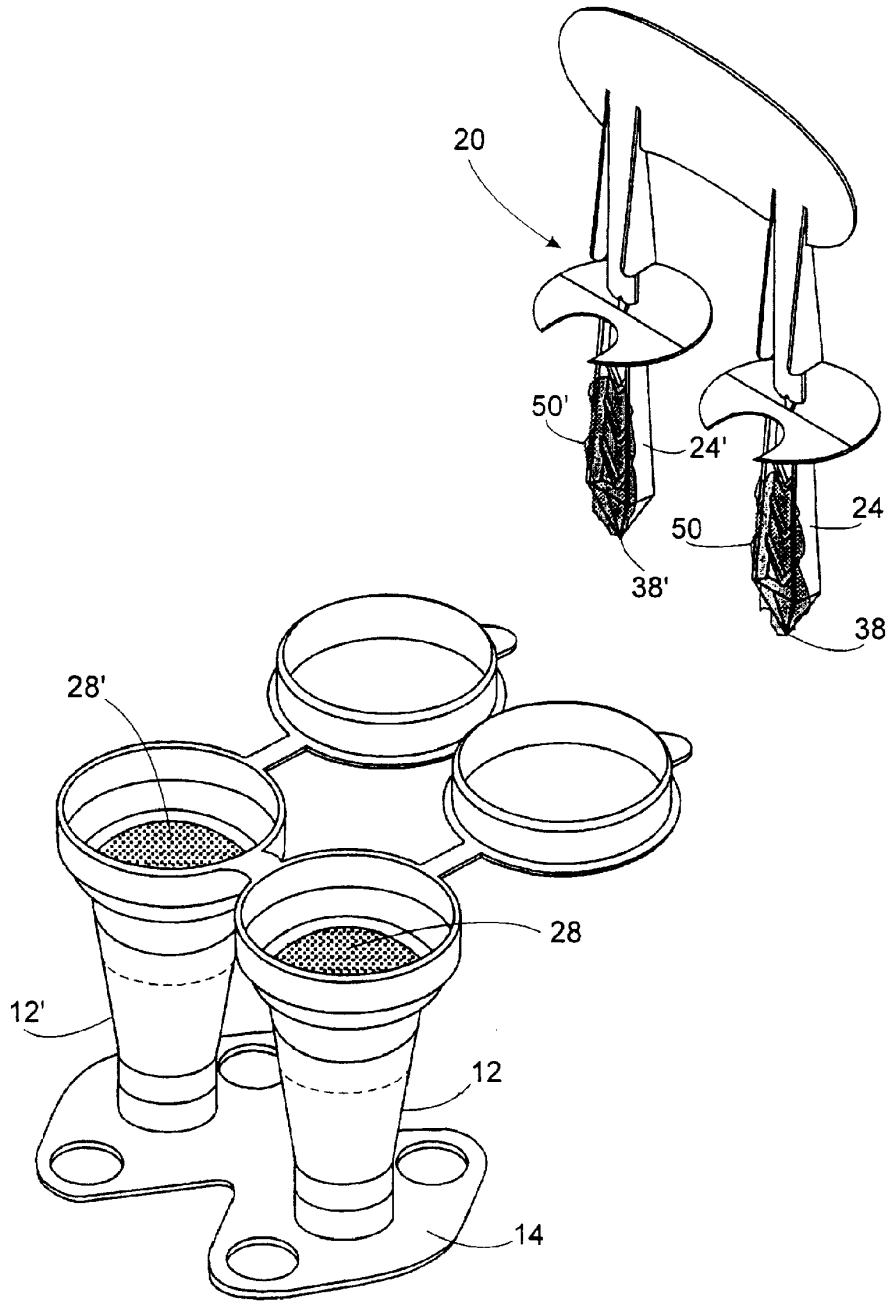


Fig. 2

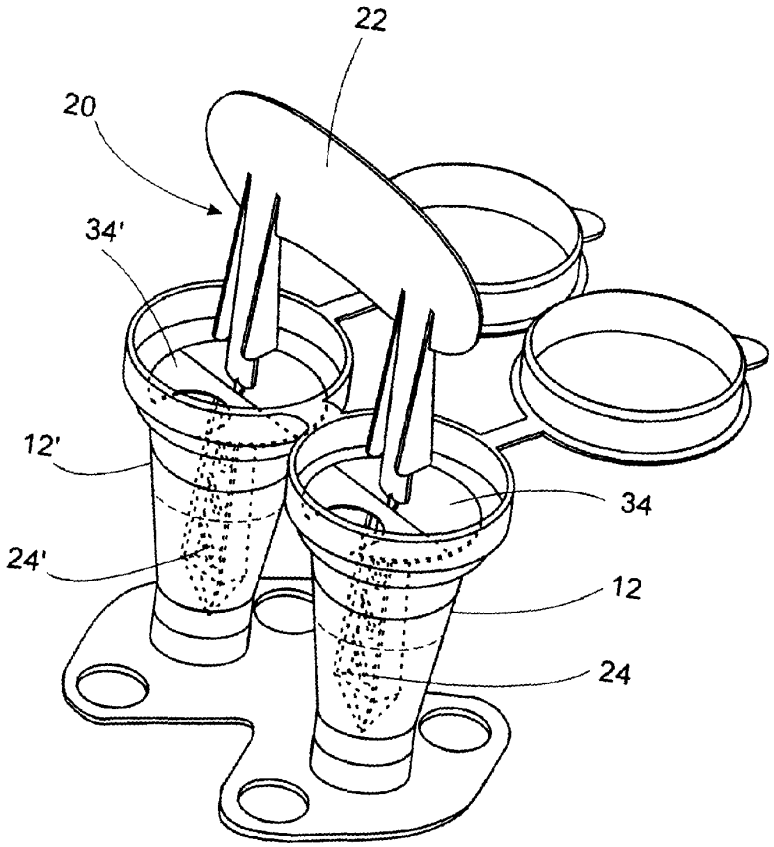


Fig. 3

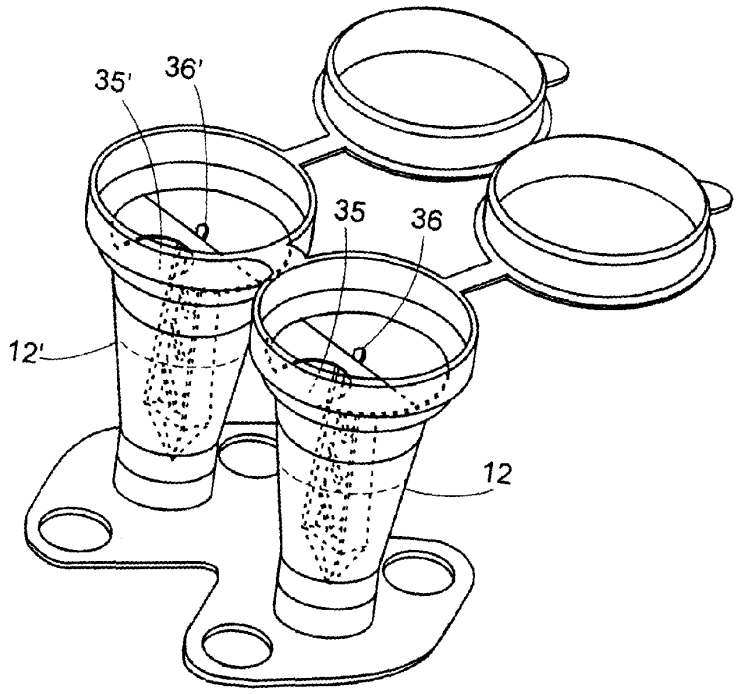


Fig. 4

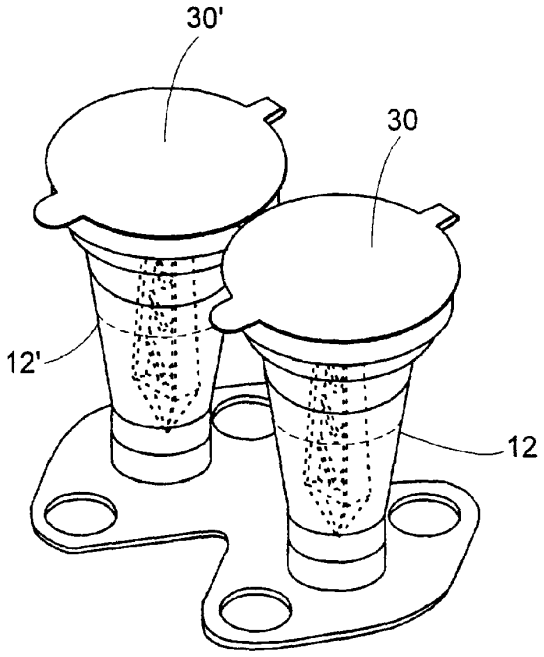


Fig. 5

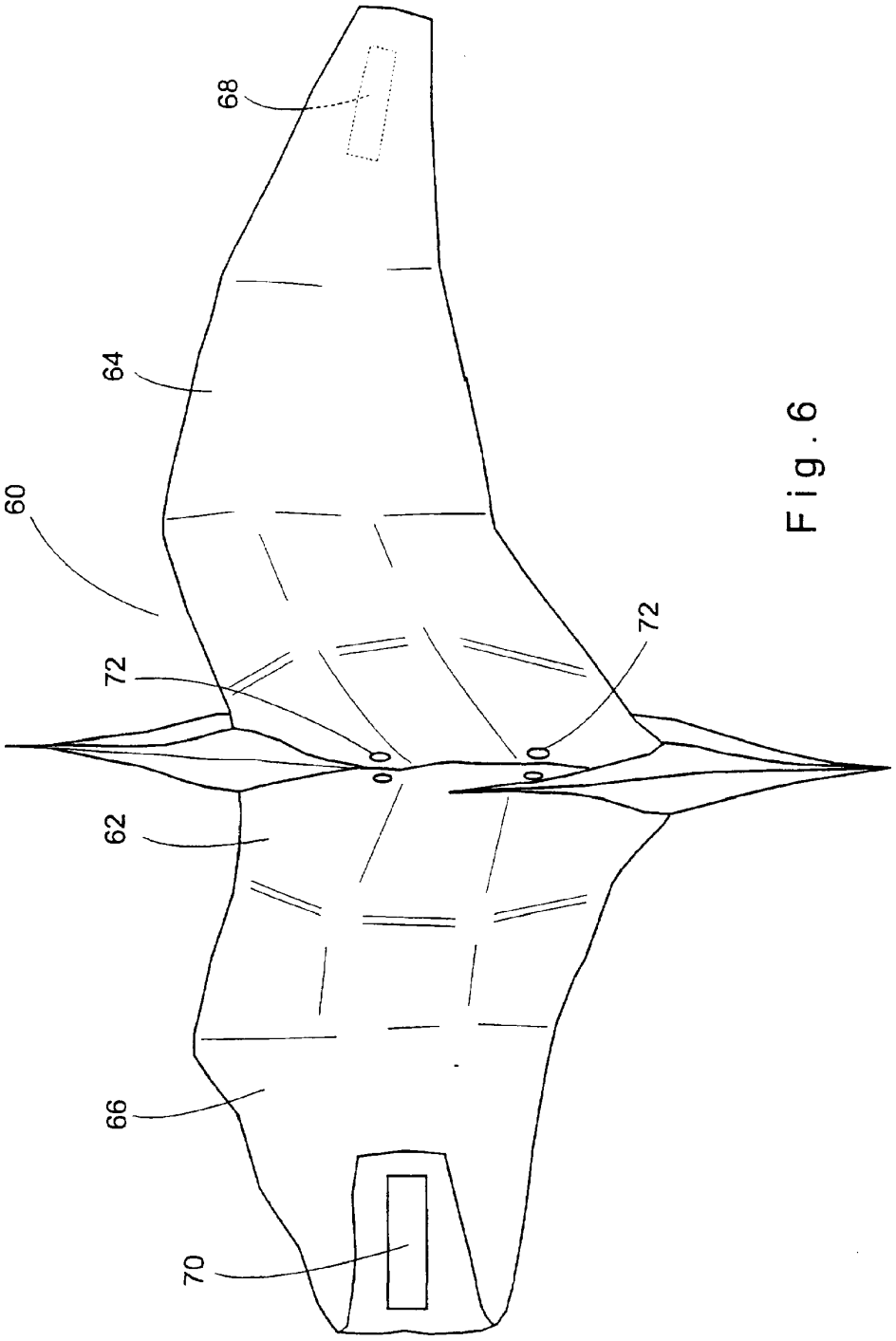


Fig. 6

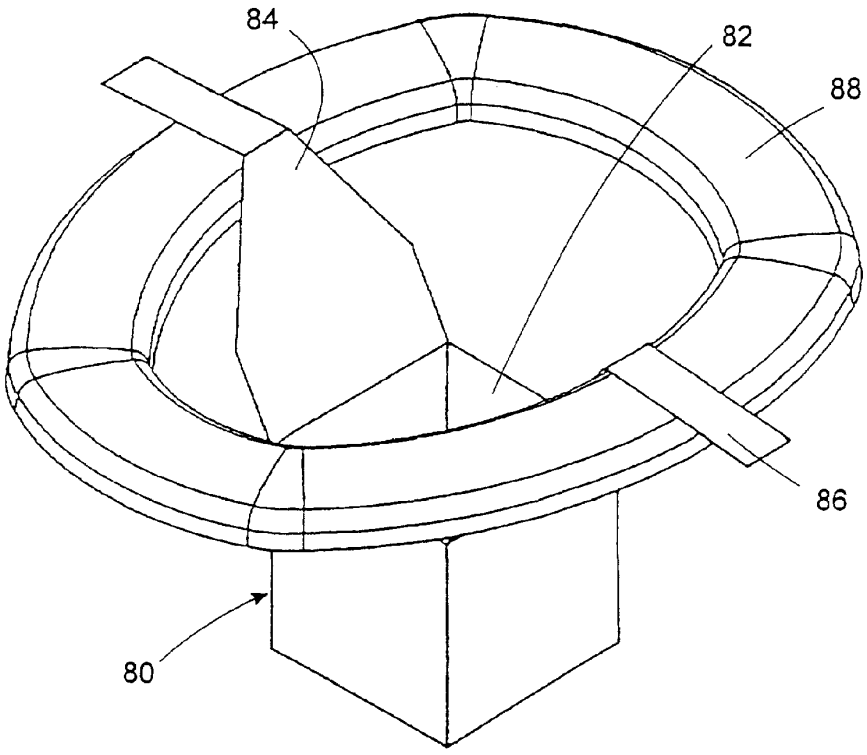


Fig. 7

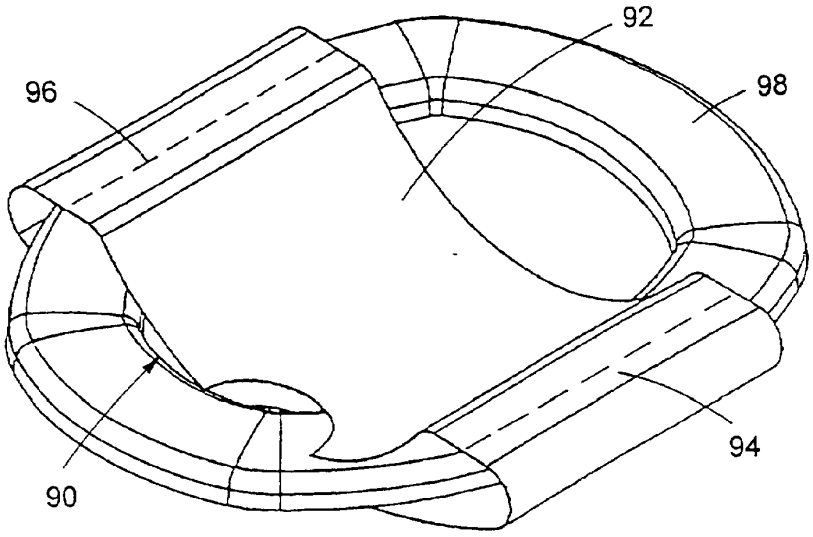


Fig. 8

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Middleton, WI 53562

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/Stephanie Filandrinos/	(Typed or printed name)
Stephanie Filandrinos	(Signature)
12/27/2023	(Date)

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

TITLE OF INVENTION:

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
CON	LE	$1200	$0.00	$0.00	$1200	02/26/2024

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

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

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

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

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(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. Casimir Jones, S.C.
2. Mary Ann D. Brow
3.

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

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Exact Sciences Corporation	Madison, WI

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

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Authorized Signature /Mary Ann D. Brow/ Date 12/27/2023

Typed or printed name Mary Ann D. Brow Registration No. 42,363

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

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

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

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

COMMENTS REGARDING EXAMINER'S STATEMENTS ON REASONS FOR ALLOWANCE MAILED 24 NOV 2023 AND CORRECTED NOTICE OF ALLOWABILTY MAILED 20 DEC 2023

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

Applicant is filing the following comments in response to the Examiner's Statement of Reasons for Allowance included with the Notice of Allowance mailed 24 NOV 2023 ("NOA"), and comments made in the Corrected Notice of Allowability mailed 20 DEC 2023 ("CNOA") for the above-referenced application. Applicant agrees with the Examiner's conclusion that claims 1-2 and 4-20 are allowable.

Applicant notes that page 2 of the NOA, second paragraph, includes the text reproduced below:

> ▶ A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed on 10 JUL 09 in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Claim(s) 27-41 is/are now pending.

(NOA, p2, 2nd para.)

Applicant notes with appreciation that, in the CNOA AT p2, 1st paragraph, the Examiner made the following correction:

> ▶ In the original NOA mailed 24 NOV 2023, the examiner mistakenly referenced an RCE, see 2nd para on p.2. This was a typographical error no RCE was filed in 18/179,945.

(CNOA, p2, 1st para.)

Regarding the NOA, p2, 2nd paragraph reproduced above, Applicant notes that Claims 27-41 are not and have never been pending in the present application and it is Applicant's position that the entirety of this paragraph was included in the NOA in error.

It is further Applicant's position that Examiner's statement in the NOA as originally mailed and as corrected by the Examiner's statement in CNOA, p2, 1st paragraph has no bearing on the allowability, scope, interpretation, or any other aspect of any of allowed claims 1-2 and 4-20 of the instant application. Applicant expressly reserves the right to set forth additional and/or alternative reasons for the patentability of the claims in future applications and/or proceedings.

It is recognized that, in accordance with M.P.E.P. § 1302.14, the Examiner's reasons for allowance need not set forth all of the details as to why the claims are allowed. In the above-referenced application, it is not conceded that the Examiner's stated reasons for allowance are the only reasons for which the claims are allowable. The Examiner's reasons for allowance indicate that particular claim elements are not disclosed or suggested by the prior art of record, yet the claims may be patentable for other reasons as well, including the inventive combination of all of the recited claim elements. Moreover, the Examiner does not assert, and it would not be conceded, that the Examiner's reasons have any bearing on the patentability of claims in any other applications directed to the disclosed subject matter. In addition, each dependent claim stands on its own and may be allowable on its own merits. In particular, each dependent claim may be allowable on the basis of a combination of some of the features recited in the dependent claim and its base claim(s), which combination of features may not include all of the limitations identified in the Examiner's reasons for allowance.

Respectfully submitted,

Dated: December 27, 2023

/Mary Ann D. Brow/

Mary Ann D. Brow
Registration No. 42,363

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

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**12/27/2023 06:07:37 PM Z ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	63767643	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 2

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-12-27_35239.307_CommentsonAllowance.pdf	3	Post Allowance Communication - Incoming	200 KB
2023-12-27_35239.307_IssueFeeTransmittal.pdf	2	Issue Fee Payment (PTO-85B)	281 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)

2023-12-27_35239.307_CommentsonAllowance.pdf	61B9613270DCDE60A103196F5D8D428262A174ACFEF78865C00300AA7AE4EC84AB2CA5285D6D00864C1DF9FF57F3F349BF9D03ED0EA85AFB9E61904F0D227051
2023-12-27_35239.307_IssueFeeTransmittal.pdf	4A99160B191127D7C87F338B56B8702BF7BD6FFC627F191650693E1B1F56EFE0BEE36F1E8F5990A8A963D49B1581603B99D85C813A0286BB425C60B30816F8F6

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	12/27/2023 06:07:37 PM Z ET	EXCTD-35239.307

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	63767643	AUTHORIZED BY	Mary Ann Brow
CUSTOMER #	173254	FILING DATE	03/07/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Joost Louwagie

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0638	E2023BQI07525006	Stephanie Filandrinos

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1501	UTILITY ISSUE FEE	1200.00	1	1200.00
			TOTAL AMOUNT:	$1,200.00

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

New Applications Under 35 U.S.C. 111

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

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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
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307	2966

173254 7590 12/20/2023

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

EXAMINER	
WHISENANT, ETHAN C	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
12/20/2023	ELECTRONIC

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

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

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

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

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

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

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

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

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

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

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

   Certified copies:

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

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

   * Certified copies not received: _____ .

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

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

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

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

Attachment(s)

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

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634

Corrected Notice of Allowability

► In the original NOA mailed 24 NOV 2023, the examiner mistakenly referenced an RCE, see 2nd para on p.2 This was a typographical error **no** RCE was filed in 18/179,945.

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

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

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

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

UNITED STATES PATENT AND TRADEMARK OFFICE

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

173254 7590 11/24/2023

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

EXAMINER	
WHISENANT, ETHAN C	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 11/24/2023

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

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	02/26/2024

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

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

HOW TO REPLY TO THIS NOTICE:

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

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

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

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

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

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

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

PTOL-85 (Rev. 11/23)

PART B - FEE(S) TRANSMITTAL

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

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

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

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

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

173254 7590 11/24/2023
CASIMIR JONES, S.C.
2275 DEMING WAY, SUITE 310
MIDDLETON, WI 53562

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

Certificate of Mailing or Transmission

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

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

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

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	02/26/2024

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

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

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

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

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

1_____
2_____
3_____

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

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

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

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

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

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

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

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

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

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

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

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

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

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

Exact Sciences Ex. 2005, Page 536 of 760

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

173254 7590 11/24/2023

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

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

DATE MAILED: 11/24/2023

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

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

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

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

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

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

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

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (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.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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

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

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

1. ☑ This communication is responsive to 06 OCT 2023.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

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

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

   Certified copies:

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

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

   * Certified copies not received: _____ .

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

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

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

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

Attachment(s)

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

/ETHAN C WHISENANT/
Primary Examiner, Art Unit 1634

NOTICE OF ALLOWABILITY

► **Claim(s) 1-2 and 4-20** as presented in the paper(s) filed 06 OCT 2023 is/are pending in this application.

► A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed on 10 JUL 09 in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Claim(s) 27-41 is/are now pending.

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

REASON(S) FOR ALLOWANCE

► **Claim(s) 1-2, and 4-20** is/are deemed to be allowable in light of the paper(s) filed 06 OCT 2023 and the persuasive argument(s) therein. The Claims have now been renumbered as Claims 1-19 in the same order as presented by the applicant.

Conclusion

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

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

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

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

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

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

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

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

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

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

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

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

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

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

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

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	19	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	16 November 2023	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20231116

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

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

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

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

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 19	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634 (Primary Examiner)	16 November 2023 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

U.S. Patent and Trademark Office

Part of Paper No.: 20231116

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

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

CLAIMS

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

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	19	
/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	16 November 2023	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20231116

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

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

CLAIMS

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

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

Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
* 18/179,945 *	18/179,945	Louwagie, Joost	
	Examiner	Art Unit	
	WHISENANT, ETHAN C	1634	

Document Code - DISQ	Internal Document - DO NOT MAIL

TERMINAL DISCLAIMER	☑ APPROVED	☐ DISAPPROVED
Date Filed: **06 October 2023**	**This patent is subject to a Terminal Disclaimer**	

Approved/Disapproved by:

2/Tds both approved.
/LAWANA R HIXON/

Technology Center: OPLC

Telephone: (571)272-6074

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**10/06/2023 03:19:09 PM ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Mary Ann Brow
PATENT CENTER #	62944184	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 5

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
2023-10-06_TermDisclPatent_EXCTD-003USCON4-35239_35239-307.pdf		2	Terminal Disclaimer Filed	237 KB
2023-10-06_TermDisclAppln_EXCTD-003USCON4-35239_35239-307.pdf		2	Terminal Disclaimer Filed	312 KB
2023-10-06_ROA_EXCTD-003USCON4-35239_35239-307.pdf		7	-	194 KB
2023-10-06_ROA_EXCTD-	(1-1)	1	Amendment/Request for	135 KB

003USCON4-35239_35239-307-A....pdf			Reconsideration-After Non-Final Rejection	
2023-10-06_ROA_EXCTD-003USCON4-35239_35239-307-CLM.pdf	(2-5)	4	Claims	175 KB
2023-10-06_ROA_EXCTD-003USCON4-35239_35239-307-REM.pdf	(6-7)	2	Applicant Arguments/Remarks Made in an Amendment	136 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
2023-10-06_TermDisclPatent_EXCTD-003USCON4-35239_35239-307.pdf	64B31EFA8290277FC16AE7DD2DCF9D7712680AC1C4D8B28194A4BA8061621719737007 25E0CAA0C9D929898145DA2191A20FEAC836295790ABE932F00DD751CB
2023-10-06_TermDisclAppln_EXCTD-003USCON4-35239_35239-307.pdf	7FFD461407661EA30F547A8088AA7AD4DC0033295CDF22671F418173F93B011CAB68F9AD1AAD26D55AF9F7A66BCF70CD2F6413ED5D42EDCC48E377F0650C8EA4
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2023-10-06_ROA_EXCTD-003USCON4-35239_35239-307-CLM.pdf	4B9726E49A040FC769852FA968AF7A2A7423D8CB0CBE48DED3EB09C33346EB63E2DB22FF6C5A43887B065C883531B242BB7B1DE0AFF26883A903563517B4C7FB
2023-10-06_ROA_EXCTD-	7C047E5C0AA843C23666ABE4CD0B7110886FB8275C986C8DB

003USCON4-35239_35239-307-REM.pdf	8C876BE3D4D037BF948EBF0B12AD91B30F393EE3AE0ABDA70AA811E5EA86F256124DB83D37A742D

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PTO/AIA/25 (04-13)
Approved for use through 05/31/2024. 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.

TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	Docket Number (Optional) EXCTD-35239.307

In re Application of: Joost Louwagie

Application No.: 18/179,945

Filed: 7 March 2023

For: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

The applicant, Exact Sciences Corporation, owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending **reference** Application Number 18/179,961, filed, 7 March 2023, as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the **reference** application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

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

Check either box 1 or 2 below, if appropriate.

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

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

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

/Mary Ann D. Brow/	10/06/2023
Signature	Date
Mary Ann D. Brow	
Typed or printed name	
Attorney	608-662-1277
Title	Telephone Number

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

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

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Additional Uses

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

AMENDMENT AND RESPONSE TO NON-FINAL OFFICE ACTION MAILED 07/06/2023

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

This paper is responsive to the Office Action mailed 07/06/2023, with response due by 10/06/2023. Applicant respectfully requests reconsideration in view of the remarks hereinbelow.

The Commissioner is authorized by this paper to charge any fees during the entire pendency of this application, including fees due under 37 C.F.R. §§ 1.16 and 1.17 that may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-4302, referencing Attorney Docket No.: **EXCTD-35239.307**. This paragraph is a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Amendment to the Claims begins on Page 2;

Remarks begin on Page 6.

AMENDMENTS TO THE CLAIMS:

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

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

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

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

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

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

3. (canceled)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

REMARKS

Information Disclosure Statements

Claims 1-20 are pending in the present application. Applicant notes with appreciation that the Examiner has considered the references disclosed by the Applicant in the Information Disclosure Statements filed in this matter on 03/07/2023 and 06/08/2023.

Claim Amendments

For business reasons and without acquiescing to Examiner arguments, and reserving the right to prosecute the original or similar claims in one or more future applications, Applicant herein cancels claim 3 without prejudice.

Office Action

In an Office Action mailed 07/06/2023, the Examiner provisionally rejected all claims on the grounds of non-statutory obviousness-type double patenting over claims 1-19 of co-pending Application Ser No. 18/179,961.

The Examiner additionally rejects Claims 1-20 over claims 1-20 of U.S. Patent No. 11,634,781.

Solely in the interest of expediting prosecution, Applicant herewith files terminal disclaimers over Application Ser. No. 18/179,961 and US Patent No. 11,634,781 to obviate the present rejections. Applicant notes that the filing of a terminal disclaimer to obviate a rejection based on non-statutory double patenting is not an admission of the propriety of the rejection.

CONCLUSION

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

Dated: October 6, 2023

/Mary Ann D. Brow/

Mary Ann D. Brow
Registration No. 42,363

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

PTO/AIA/26 (04-14)
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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) EXCTD-35239.307

In re Application of: Joost Louwagie

Application No.: 18/179,945

Filed: 7 March 2023

For: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

The applicant, Exact Sciences Corporation, owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of **prior patent** No. 11,634,781 as the term of said **prior patent** is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

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

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

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

/Mary Ann D. Brow/ — Signature
10/06/2023 — Date

Mary Ann D. Brow — Typed or printed name

Attorney — Title
608-662-1277 — Telephone Number

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included.

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**10/06/2023 03:19:09 PM ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Mary Ann Brow
PATENT CENTER #	62944184	AUTHORIZED BY	Mary Ann Brow
CUSTOMER #	173254	FILING DATE	03/07/2023
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Joost Louwagie

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0638	E202306F19279874	Stephanie Filandrinos

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1814	STATUTORY DISCLAIMER, INCLUDING TERMINAL DISCLAIMER	170.00	2	340.00
			TOTAL AMOUNT:	**$340.00**

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

New Applications Under 35 U.S.C. 111

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

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New International Application Filed with the USPTO as a Receiving Office

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
18/179,945	03/07/2023	Joost Louwagie	EXCTD-35239.307

CONFIRMATION NO. 2966

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

PUBLICATION NOTICE

OC000000062913983

Date Mailed: 10/05/2023

Title:FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Publication No.US-2023-0313317-A1
Publication Date:10/05/2023

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The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

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In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

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

173254 7590 07/06/2023

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2275 DEMING WAY, SUITE 310
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EXAMINER	
WHISENANT, ETHAN C	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
07/06/2023	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.

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Office Action Summary	**Application No.** 18/179,945	**Applicant(s)** Louwagie, Joost	
	Examiner Ethan C Whisenant	**Art Unit** 1634	**AIA (FITF) Status** No

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

Period for Reply

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

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

Status

1)☑ Responsive to communication(s) filed on 07 MAR 2023.

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

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

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

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

Disposition of Claims*

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

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

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-20 is/are rejected.

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

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

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

Application Papers

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

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

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

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

Priority under 35 U.S.C. § 119

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

Certified copies:

a)☑ All b)☐ Some** c)☐ None of the:

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

2.☑ Certified copies of the priority documents have been received in Application No. 13147570.

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 MAR 2023.

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

4) ☐ Other: _____.

Detailed Action

► **Claim(s) 1-20** as presented in the paper(s) filed 07 MAR 2023 is/are pending.

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

Non-Statutory Obviousness-type Double Patenting

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

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

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should

be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Non-Statutory Obviousness-type Double Patenting Rejections

► **Claim(s) 1-20** is/are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-19 of copending Application No.18/179,961. Although the conflicting claims are not identical, they are not patentably distinct**. This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented**.

► **Claim(s) 1-20** is/are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-20 of U.S. Patent No. 11,634,781 (hereinafter "US -781'").

Although the conflicting claims are not identical, they are not patentably distinct from each other. For example, Claim 1 of US-781 teach a method of processing a freshly-collected fecal sample without freezing that comprises collecting a fecal sample from a human subject, wherein the fecal sample is collected at home by the human subject; in a sealable vessel, combining a first portion of the fecal sample with a stabilizing buffer, and sealing the sealable vessel; and in a sealable container, combining a second portion of the fecal sample with a solution that prevents denaturation or degradation of blood proteins found in a fecal sample, and sealing the sealable container.

Prior Art

► The Claims are allowable over the prior art of record because the prior art of record fails to teach dividing a feces/stool sample collected at home into at least two portions. One portion of which is combined with a 1st stabilizing buffer (i.e. nucleic acid stabilizing buffer) while the second portion is combined with a solution that prevents denaturation or degradation of blood proteins found in the fecal sample. The closest prior art is considered to be Lapidus et al. [US 5,952,178 – hereinafter "Lapidus"] - cited by applicant. However, Lapidus does not teach or reasonably suggest dividing a feces sample collected at home into at least two portions. One portion of which is combined with a 1st stabilizing buffer (i.e. nucleic acid stabilizing buffer) while the second portion is combined with a solution that prevents denaturation or degradation of blood proteins found in the fecal sample. The teaching of Lapidus alone or in combination with the other prior art of record would have suggested to PHOSITA to place the a stool, or a portion thereof, collected at home into a sealable container and to combine said stool or portion thereof with a stabilizing buffer which stabilizing buffer stabilizes both nucleic acids and proteins found within the stool sample or portion thereof. The claims clearly require the division of the stool into two separate and distinct samples. A first for nucleic acid analysis and a second for protein analysis. The prior art of record fails to teach this feature.

Conclusion

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

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

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

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

EXAMINER SEARCH NOTES

28 JUN 2023 - ECW

Databases searched: All available via PE2E SEARCH
CAplus, Medline and BIOSIS via STNext; and **Google Scholar** (note the search terms used below)

Reviewed the parent(s), if any, and any search(es) performed therein : see the BIB data sheet

Reviewed, the search(es), if any, performed by prior examiners including any international examiners. Reviewed the Search Report and Written opinion in PCT/GB2010/000180

Planned Search

Search terms:

All Inventor(s) e.g. Louwagie?/au
Feces or faeces or stool
Process$
Collect$
divid$ or partion$ or portion$ or separat$
sealable (vessel$ or container$)
blood proteins
colorectal cancer or CRC

► See the Examiner's PE2E SEARCH notes/strategy in IFW

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

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A					
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N	JP-4039590-B2	01-2008	JP	Yoshizawa et al.	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Nechvatal et al. Journal of Microbiological Methods 72: 124-132 (Year: 2008)
	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.

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

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

CLAIMS										
☐ Claims renumbered in the same order as presented by applicant					☐ CPA		☐ T.D.		☐ R.1.47	
CLAIM		**DATE**								
Final	Original	06/28/2023								
	1	✓								
	2	✓								
	3	✓								
	4	✓								
	5	✓								
	6	✓								
	7	✓								
	8	✓								
	9	✓								
	10	✓								
	11	✓								
	12	✓								
	13	✓								
	14	✓								
	15	✓								
	16	✓								
	17	✓								
	18	✓								
	19	✓								
	20	✓								

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

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

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

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

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

Search Notes		
Search Notes	**Date**	**Examiner**
See the Examiner's Search Notes	06/28/2023	ECW

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

/ETHAN C WHISENANT/ Primary Examiner, Art Unit 1634	

Bibliographic Data

Application No: 18/179,945

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /ETHAN C WHISENANT/ Examiner's Signature | Initials

Title: FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
03/07/2023	435	1634	EXCTD-35239.307
RULE			

APPLICANTS

Exact Sciences Corporation, Madison, WI, UNITED STATES

INVENTORS

Joost Louwagie, Dornach, SWITZERLAND

CONTINUING DATA

This application is a CON of 17936335 09/28/2022 PAT 11634781

17936335 is a CON of 15634607 06/27/2017

15634607 is a CON of 15010436 01/29/2016ABN

15010436 is a CON of 13147570 03/12/2012ABN

13147570 is a 371 of PCT/GB2010/000180 02/03/2010

PCT/GB2010/000180 has PRO of 61149581 02/03/2009 *

(*) Data provided by applicant is not consistent with PTO record

FOREIGN APPLICATIONS

PCT/GB2010/000180 02/03/2010

IF REQUIRED, FOREIGN LICENSE GRANTED**

05/19/2023

STATE OR COUNTRY

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

FILING FEE RECEIVED

$6,160

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	1	of	2	Attorney Docket Number	EXCTD-35239.307

U.S. PATENTS					
Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		4859610	1989-08-22	MAGGIO	
		5137806	1992-08-11	LEMAISTRE et al.	
		5196167	1993-03-23	GUADAGNO et al.	
		5527676	1996-06-18	VOGELSTEIN et al.	
		5741650	1998-04-21	LAPIDUS et al.	
		5952178	1999-09-14	LAPIDUS et al.	
		11634781	2023-04-25	LOUWAGIE	

U.S. PUBLISHED PATENT APPLICATIONS					
Examiner Initials*	Cite No.[3]	Document Number Number-Kind Code[4] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

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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]
		EP 1366715	2003-12-03	SENTINEL CH. S.r.L		
		WO 2005/014154	2005-02-17	LOOTS LIMITED		

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]

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	06/28/2023

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

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		Request for Ex Parte Reexamination of U.S. Patent 11,634,781, filed May 22, 2023, 286 pages	
		Declaration of Mr. Anthony P. Shuber, MS, Reexam Application 90/015,237, filed May 22, 2023, 63 pages	

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CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

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

SIGNATURE

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

Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2023-06-08
Name/Print	Mary Ann D. Brow	Registration Number	42363

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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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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
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				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
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U.S. PATENTS					
Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		4437975	1984-03-20	GILLESPIE et al.	
		4445235	1984-05-01	SLOVER et al.	
		4582811	1986-04-15	PUCCI et al.	
		4683197	1987-07-28	GALLATI	
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		6761702	2004-07-13	SMITH	
		7288413	2007-10-30	GOULDEN	
		7371527	2008-05-13	BAYLIN et al.	
		8969046	2015-05-03	VAN ENGELAND et al.	

U.S. PUBLISHED PATENT APPLICATIONS					
Examiner Initials*	Cite No.[3]	Document Number Number-Kind Code[4] (if known)	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20020187476	2002-12-12	KOROULIS et al.	
		20020096469	2002-07-25	FAULKNER	
		20030086869	2003-05-08	STALLINGS	
		20030096244	2003-05-22	RABELLO et al.	
		20040019298	2004-01-29	ZHOU et al.	
		20040091881	2004-05-13	OLEK et al.	
		20050064401	2005-03-24	OLEK et al.	
		20050075543	2005-04-07	CALABRESE	
		20050244836	2005-11-03	TSANG	
		20060084054	2006-04-20	ALSOBROOK et al.	
		20060188939	2006-08-24	GAO	
		20060210448	2006-09-21	WANG et al.	

Examiner Signature	/ETHAN C WHISENANT/	Date Considered	06/28/2023

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				Art Unit	
				Examiner Name	
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		20060216830	2006-09-28	KIKUIRI	
		20070017015	2007-01-25	FINELL	
		20070072214	2007-03-29	GARVIN	
		20080097238	2008-04-24	LOKTIONOV et al.	
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		20160194723	2016-07-07	LOUWAGIE	
		20230048152	2023-02-16	LOUWAGIE	

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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]
		EP 0032782	1981-07-29	KOMMANDIITTIYHTIO FINNPIPETTE		
		EP 0308227	1989-03-22	SMITHKLINE DIAGNOSTICS, INC.		
		EP 0817968	1998-01-14	EXACT SCIENCES CORPORATION		
		FR 2919065	2009-01-23	BIOMERIEUX		
		WO 1990/06995	1990-06-28	SISKA DIAGNOSITCS, INC.		
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		WO 2000/050640	2000-08-31	EXACT LABORATORIES, INC.		
		WO 2004/067726	2004-08-12	KECK GRADUATE INSTITUTE		

Examiner Signature		Date Considered	

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				Art Unit	
				Examiner Name	
Sheet	3	of	5	Attorney Docket Number	EXCTD-35239.307

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		WO 2004/0927209	2004-10-28	KIETURAKIS & KARCZEWSKI		
		WO 2005/014154	2005-02-17	LOOTS LIMITED		
		WO 2005/017207	2005-02-24	CASE WESTERN RESERVE UNIVERSITY		
		WO 2006/113671	2006-10-26	ONCOMETHYLOME SCIENCES SA		
		WO 2008/010975	2008-01-24	THE JOHNS HOPKINS UNIVERSITY		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		
		WO 2008/100913	2008-08-21	THE JOHNS HOPKINS UNIVERSITY		
		WO 2008/102002	2008-08-28	RIKSHOSPITALET RADIUMHOSPITALE		

NONPATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and-or country where published.	Translation[6]
		ABBASZADEGAN et al., Stool-based DNA testing, a new noninvasive method for colorectal cancer screening, the first report from Iran. World J Gastroenterol. 2007 Mar 14;13(10):1528-33	
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		CONROY et al., Exact Sciences Completes 40,000 Cologuard Tests During First Quarter 2016, Exact Sciences Latest News, Pages 1-10, 05/03/2016.	

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				Art Unit	
				Examiner Name	
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		EXACT SCIENCES, Cologuard(TM) sDNA-based Colorectal Cancer Screening Test - Instructions for Use, Pages 1-71, 2013.	
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NONPATENT LITERATURE DOCUMENTS			
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Scope of Claims Close

(57) [Claim]
[Claim 1]
To provide a method for stabilizing a hemoglobin sample by coexisting a partial decomposition product of hemoglobin by a proteolytic enzyme in an immunological hemoglobin measurement using an antibody recognizing mammalian hemoglobin.
[Claim 2]
The method for stabilizing a hemoglobin sample according to claim 1, wherein the proteolytic enzyme is trypsin, pepsin or alkalase.
[Claim 3]
The method for stabilizing a hemoglobin sample according to claim 1 or 2, wherein the hemoglobin sample is present in a fecal suspension, and the dispersion medium of the suspension contains a partial decomposition product of hemoglobin by a proteolytic enzyme.
[Claim 4]
A method for stabilizing a hemoglobin sample according to claim 1 or 2, wherein the hemoglobin sample is present in urine and a partial decomposition product of hemoglobin by a proteolytic enzyme is added to the urine.
[Claim 5]
A method of stabilizing a hemoglobin sample according to claims 1 to 4, wherein the hemoglobin sample is human hemoglobin.
[Claim 6]
A dispersion medium for suspending a fecal sample to be tested for the presence of hemoglobin, the dispersion medium comprising:

Dispersion medium containing a partial degradation product of hemoglobin by a leukolytic enzyme
[Claim 7]
The dispersion medium according to claim 6, wherein 10 to 200 μ g of a partial decomposition product of hemoglobin by a proteolytic enzyme is added per fecal 1mg to be suspended.
[Claim 8]
The dispersion medium according to claim 7, wherein the concentration of the partial degradation product of hemoglobin by the proteolytic enzyme in the dispersion medium is 1 μ g/ml or more.
[Claim 9]
The method according to claim 1, wherein the immunological measurement is carried out by immunological aggregation of insoluble carrier particles to which an antibody recognizing human hemoglobin is bound.
[Claim 10]

The measurement method according to claim 9, wherein a mixture of insoluble carrier particles to which a monoclonal antibody r ecognizing human hemoglobin is bound and insoluble carrier particles to which a polyclonal antibody recognizing human hemogl obin is bound is used.

[Claim 11]

The method according to claim 1, wherein the immunoassay is immunochromatography using, as a marker, colored insoluble car rier particles to which a monoclonal antibody that recognizes human hemoglobin is bound.

Detailed Description Close

[Detailed description of the invention]

[0001]

[Industrial field of application]

The present invention relates to a method for stabilizing hemoglobin. Specifically, the present invention relates to a technique for stabilizing hemoglobin in a sample to be detected or hemoglobin as a standard substance used as a positive control. Detection of hemoglobin contained in urine, feces and the like is useful for diagnosis of many diseases. In particular, the detection of hemoglo bin in feces (fecal occult blood) is important information in the diagnosis of digestive diseases including colon cancer. In place of a test paper method based on a chemical color development reaction which has been used for hemoglobin in feces from old time s, in recent years, a detection method by an immunological technique using an antibody specific to human hemoglobin has beco me widespread and has been established as a convenient test method which does not require dietary restriction.

[0002]

[Problems of Related Art]

In order to detect hemoglobin contained in a sample such as feces, it is necessary to transport the sample to a testing facility. A transport container [1] provided with a feces collection mechanism and a feces suspension filtration mechanism is used for trans porting a feces sample. By using this type of container, it is possible to quantitatively collect feces, and it is possible to easily filte r a feces suspension. The container from which the feces has been collected is transported to an inspection facility by means of mailing or the like.

[0003]

During transportation, hemoglobin coexists with bacteria and many other components contained in feces. Further, since it is gen erally difficult to control the temperature, it is inevitable that the product is exposed to a temperature condition which is not pref erable for storage. Thus, there is always the possibility of denaturation and degradation of hemoglobin during transport. Since de naturation and decomposition of hemoglobin during transportation lead to erroneous diagnostic results, it is desirable to minimiz e such denaturation and decomposition.

[0004]

In order to stabilize not only hemoglobin but also protein substances, it is usually effective to add other proteins or sugars. With respect to hemoglobin, it is known that bovine serum albumin (hereinafter abbreviated as BSA), rabbit serum albumin, ovalbumi n or the like as a protein and sucrose or the like as a sugar exhibit a stabilizing effect [2]. The use of animal serum has also bee n reported [3]. However, these general stabilizers have a small hemoglobin stabilizing effect particularly in a fecal suspension, an d sufficient storage performance cannot be expected. Many components such as bacteria and proteolytic enzymes which cause d enaturation and decomposition of hemoglobin are present in feces, and hemoglobin cannot be sufficiently protected only by prot ein and sugar. Further, since animal serum is not a purified pure substance, it has a stabilizing effect.

It is easy to produce a ** difference. In addition, since it cannot be denied that there is a possibility that a component having a denaturation effect on hemoglobin is present in animal serum containing many components, it is hard to say that the stabilizer is a desirable stabilizer. Further, in a report [3] in which stabilization of hemoglobin was attempted with bovine serum, it was neces sary to add a large amount of serum such as 10 to 20% v/v. Stabilization techniques that require large amounts of expensive an imal serum are economically disadvantageous.

[0005]

Techniques for stabilizing hemoglobin in fecal suspensions include the addition of lytic enzymes [4], the use of antimicrobial com

pounds and the like [5] [6], the addition of animal hemoglobin [7] Addition of protease inhibitors [8], control of pH [9], addition of iron protoporphyrin [10], iron-containing proteins such as transferrin [11] [15] and peroxidase [12], and addition of sodium fl uoride [14] are known. Alternatively, multi-component combinations [13] have also been tried. It is considered that these inhibit the influence of bacteria or disperse the influence on hemoglobin by a compound structurally similar to hemoglobin, thereby exhi biting a protective effect on hemoglobin.

[0006]

In addition, it is known that ethylenediaminetetraacetic acid (hereinafter abbreviated as EDTA) has a hemoglobin stabilizing effec t [16]. However, as a result of additional tests by the present inventors, it was confirmed that EDTA cannot be expected to have a sufficient stabilizing effect on hemoglobin in feces. The Applicant has filed a patent application on the use of a water-soluble m etal complex of a transition metal ion which has a higher stabilizing effect than simple EDTA alone [17]. In addition, technique fo r stabilizing blood protein, which utilizes the fact that antibodies against feces components contribute to the stabilization of blood protein present in feces, was also filed [18].

[0007]

Among these stabilizers, substances structurally similar to hemoglobin, for example, iron porphyrin such as heme and hemin and animal hemoglobin are particularly effective for the antigen stability of hemoglobin in immune reaction. However, heme and hem in are not sufficiently soluble in water, and it is necessary to examine a dissolution accelerator in order to incorporate them into a fecal suspension or the like in an amount sufficient for stabilization. In addition, the addition of heterologous animal hemoglobin is disadvantageous in that cross-reaction cannot be avoided and false positive is likely to occur.

[0008]

[Problem to be solved by the invention]

A first object of the present invention is to provide a novel hemoglobin protective substance. In particular, it is the most importa nt object of the present invention to provide a technique for effectively stabilizing hemoglobin which coexists with feces compone nts having a strong denaturation and decomposition action.

A second object of the present invention is to provide a technique for stabilizing hemoglobin as a sample for analysis. Specificall y, an object of the present invention is to provide a technique for stabilizing hemoglobin to be detected in a method for detecting hemoglobin by an immunological method using an antibody that recognizes hemoglobin, which is currently the mainstream.

A third object of the present invention is to provide a dispersion medium for suspending a fecal sample for the purpose of immun ological analysis using a novel hemoglobin stabilizer, and a measurement technique using the same.

[0009]

[Means for solving the problem]

The present invention relates to a method for stabilizing hemoglobin by allowing an enzymatic decomposition product of hemogl obin to coexist, and a dispersion medium for suspending a fecal sample to which the stabilization method is applied. In addition, the present invention provides an immunological measurement method using an antibody that recognizes human hemoglobin co ntained in a biological sample, in which a biological sample stored in the presence of an enzymatic decomposition product of he moglobin is used as an analysis target.

[0010]

The hemoglobin to be enzymatically degraded may be human hemoglobin or heterologous animal hemoglobin. This is because a ntigenic globin is degraded by a proteolytic enzyme such as pepsin and loses its antigenicity. The time for pepsin digestion of he moglobin is suitably about 1 hour at 37 ° C. Globin fragmented below 6kD and antigenic to hemoglobin antibodies

* -- completely ** -- it is divided. When the enzyme reaction is further continued, the globin is further digested to form heme, w hereby the solubility is reduced and the auxiliary stabilizing action of the protein (globin fragment) is lost.

[0011]

The present inventors have found that a digestion product by alcalase having an activity in an alkaline condition, which is opposit e to pepsin having an activity in an acidic condition, also has substantially the same effect as that of a pepsin digestion product. The hemoglobin decomposition product obtained by this method is commercially available from Asahi Kasei Corporation as HIP (Hem Iron Compound), which is an iron source having excellent absorbability.

[0012]

The method for stabilizing hemoglobin of the present invention is useful for stabilizing hemoglobin as an analyte present in a biol ogical sample. To highly stabilize the antigenicity of hemoglobin to be measured, especially in a method for measuring hemoglobi n by an immunological method using an antibody recognizing hemoglobin. Feces and urine are known as biological samples in w hich hemoglobin is to be detected. Hemoglobin in the stool is indicative of bleeding in the digestive tract, while hemoglobin in th

e urine is indicative of suspected bleeding in the urinary tract.
In particular, hemoglobin in feces is generally identified from hemoglobin derived from a meal by using an immunological metho d having high specificity, and it is highly necessary to stably maintain the antigenicity of hemoglobin.
[0013]
When the stabilization method of the present invention is applied to hemoglobin present in a stool sample, a hemoglobin degrad ation product may be added to a dispersion medium in which the stool is suspended. In the usual detection of hemoglobin in fec es, feces are suspended in an appropriate dispersion medium and filtered as necessary to prepare a sample for immunological a nalysis. It is advantageous to add a decomposition product of hemoglobin to the dispersion medium used at this time. As a resul t of an experiment, the concentration of the hemoglobin degradation product in the dispersion medium is 1 μ g/ml or more.
[0014]
On the other hand, as described above, at present, a simple transport container capable of performing collection, transport, susp ension, and filtration of feces in one container has been put into practical use. This type of container is filled with a dispersion m edium at the time of shipment, and a subject may collect feces by himself / herself and mail the container to an examination faci lity. In general, since the subject is not familiar with such a work, it is necessary to assume a case in which the amount of collect ed feces cannot be strictly controlled. Further, it is desirable to use it in an excessive amount so that there is no problem even if the hemoglobin protective action of the degradation product of hemoglobin is somewhat reduced during transportation. In order to rapidly bring hemoglobin into contact with the degradation product of hemoglobin in the dispersion medium, it is desirable to use an excess amount of hemoglobin. Therefore, for example, when it is assumed that the hemoglobin decomposition product is used for stabilization of human hemoglobin, the hemoglobin decomposition product may be added in a range of 0.1 to 100 μ g, p referably 1 to 30 μ g, with respect to the amount of 1mg feces to be collected, which is assumed at the time of design.
[0015]
In addition to the hemoglobin degradation product which is the protective agent of the present invention, known components ca n be added to the dispersion medium. For example, addition of a buffer giving a pH advantageous for preservation of hemoglobi n, an antibacterial agent for preventing unnecessary proliferation of microorganisms, or many hemoglobin protective component s known so far is also effective. For example, the protective effect of the following components is known:
Examples of the inactive protein having a hemoglobin stabilizing action include serum albumin derived from human, bovine, rabb it, horse, sheep, goat, or the like, albumin derived from egg white, and the like. Amino acids such as lysine and histidine are also found to have a protective effect on hemoglobin. Known antimicrobial agents include lytic enzymes [4], azides, ethyl benzoate, p enicillin, fungizone [5], streptomycin, or cephamycin and other non-penicillin antibiotics [6]. It is also known that protease inhibi tors such as trypsin inhibitor and α 2 macroglobulin stabilize hemoglobin [8]. Sodium fluorides [14] or water-soluble metallic co mplexes of transition metal ions such as the FeIIIEDTA complex [

17] also reports a stabilizing effect on hemoglobin. Further, salts or the like for adjusting ionic strength may be added.
[0016]
An example of a specific composition of the solution for stabilizing hemoglobin according to the present invention is shown belo w.
Degradation products of hemoglobin: 100 μ g/ml
HEPES buffer (pH7. 4): from 10 to 500mM
BSA:0. 1-5%
NaN3:0.1-1 %
[0017]
The pH of the dispersion medium is set within a range in which hemoglobin can be stably retained. Under extreme acidic or alkali ne conditions, the stability of hemoglobin may be impaired, and the digestive enzyme used for the degradation may recover its a ctivity, so that a pH in the neutral range is desirable. Specifically, the pH may be about 5 to 10, preferably about 6 to 8. A suitabl e buffer is utilized to maintain the pH. For example, hydroxylethyl piperazine-2-ethanesulfonic acid (abbreviated as N-2 Hydroxy ethylpiperazine - N' - 2 ethanesulfonic acid ,HEPES); A Good buffer such as piperazine-bis(2-ethanesulfonic acid) (Piperazine - N, N' - bis (abbreviated as 2 - ethanesulfonic acid),PIPES)) can be used in combination with A particularly preferred buffering ag ent is one which provides a pH (6 to 8) at which the structure of hemoglobin is most stabilized and which is also used as a reacti on buffer when hemoglobin is detected by an immune reaction. In addition, a phosphate buffer solution, a Tris buffer solution, a glycine buffer solution and the like can also be used.
[0018]
The method for stabilizing hemoglobin of the present invention can be used for stabilizing hemoglobin in a fecal sample for the p

urpose of detecting stools fecal occult blood. In particular, the present invention is useful for maintaining the antigenicity of hem oglobin as an immunological analyte for which protection of the antigenic structure is required.
[0019]
The present invention provides an immunological method for measuring hemoglobin using the hemoglobin stabilization techniqu e. Examples of the immunological measurement method include a latex agglutination reaction method, a gold colloid agglutinatio n reaction method, an immunochromatography method, and an ELISA method. In any of the measurement methods, the presen ce of the degradation product of hemoglobin in the hemoglobin-containing sample protects the antigen activity during storage su ppresses a decrease in the measurement value.
[0020]
It is also possible to construct an agglutination reaction system only with a monoclonal antibody. In order to carry out an aggluti nation reaction only with a monoclonal antibody, it is advantageous to combine a plurality of monoclonal antibodies that recogniz e different epitopes on hemoglobin. Hemoglobin has a tetrameric structure in which two α chains and two β chains are associate d with each other, and in principle, even one kind of monoclonal antibody aggregates. However, since hemoglobin is in an equilib rium state of a dimer structure and a tetramer structure of α β in a solution, a single monoclonal antibody cannot react with all o f them. In addition, since a single monoclonal antibody cannot react when a target epitope is denatured, it is stochastically adva ntageous to use a plurality of kinds of monoclonal antibodies in combination.
[0021]
[Action and effect]
The degradation product of hemoglobin in the present invention has an action of effectively suppressing a decrease in the measu red value of hemoglobin during the storage period. In particular, it exhibits a sufficient protective effect against hemoglobin in a f ecal suspension which contains a large amount of denaturation / decomposition components and is difficult to control the storage conditions.
It is not known by what mechanism the degradation products of hemoglobin protect hemoglobin.
[0022]
[Effect of the Invention]
According to the technique for stabilizing hemoglobin of the present invention, a protective action can be obtained even in the pr esence of a feces components having a strong denaturation / decomposition action on hemoglobin. Therefore, the part of feces o ccult blood

This method is useful for stabilizing hemoglobin contained in a sample to be precipitated. In particular, the present invention con tributes to the maintenance of antigenicity of hemoglobin as an immunological analysis target for which protection of an antigen structure is required. According to the present invention, hemoglobin in a stool sample is effectively stabilized, and prevention of false negative results due to denaturation and decomposition of hemoglobin can be expected. The enzymatic decomposition prod uct of hemoglobin required in the present invention is economically advantageous because the raw material and the enzyme use d for decomposition are inexpensive. Further, since the decomposition product of hemoglobin exhibits a high stabilizing effect ev en in a small amount, it can also be said to be an economically advantageous stabilizing technique.
The invention the present invention will be described in more detail with reference to examples.
[0023]
[Examples]
1. Production of enzymatic degradation products of animal hemoglobin
Sodium citrate was added to the swine bloods collected from the slaughtered swine to a concentration of 0.3%, and swine hemo cytes were obtained by centrifugation (6000rpm, 20 minutes). The porcine blood cells were hemolyzed by repeated freeze-thawi ng. About 8ml of conc. hydrochloric acid was added to the 1000ml of the hemolyzed blood to adjust pH1, and 50g of pepsin pow der (1200U/mg, Sigma) was added thereto, followed by gentle stirring at 30 ° C. for 1 hour, and then 1mol of NaOH was added t o neutralize the hemolyzed blood to stop the enzyme reaction. Subsequently, a fraction having a molecular weight of 5000 to 60 00 was collected using an ultrafiltration membrane (manufactured by SIP1013 Asahi Kasei Corporation) and freeze-dried.
[0024]
2. Hemoglobin Stabilization by Hemoglobin Degradation Products
The enzymatic decomposition product of bovine hemoglobin obtained in Example 1 and HIP (manufactured by Asahi Kasei Corpo ration) were each added to fecal suspensions (HEPES buffer (pH7. 4) 100mM,BSA 1%,NaN3 0.3 %) at a concentration of 100 μ g/ml, and the stabilities of the fecal suspensions alone and hemoglobin were compared.
Two above-mentioned type of buffer solution [2 ml of] was dispensed to each stool receptacle of OC-*****' Nutrition research

'(made by EIKEN CHEMICAL), human feces were extracted in the container according to the operation manual, and the amount o f hemoglobin 24 hours immediately after extraction and after 37 degree C was measured by the facilities occult blood measuring apparatus OC-sensor II (EIKEN CHEMICAL sale). The results are shown in Table 1.

[0025]

[Table 1]

糞便中ヘモグロビンの安定性

単位:ng/ml

	懸濁液のみ		牛Hb酵素分解物添加		HIP添加	
	0hr	24hr	0hr	24hr	0hr	24hr
便1	742	31	738	293	739	273
便2	747	76	745	307	755	322
便3	701	154	636	469	668	454
便4	766	133	729	433	741	420
便5	717	100	679	244	683	221
便6	705	111	695	415	690	397
便7	796	251	801	510	815	506
平均	739	122	718	382	727	370

[0026]

While the hemoglobin in the suspension alone decreased to an average of 16.5% after 24 hours at 37 ° C., the hemoglobin in th e buffer obtained by adding the enzymatic decomposition product of bovine hemoglobin to the suspension remained in an averag e of 53%, and the hemoglobin in the buffer obtained by adding HIP remained in an average of 50%. Both of them were confirme d to have an excellent stabilizing effect on the antigenicity of hemoglobin.

[0027]

3. Confirmation of cross-reactivity

Hemoglobin concentration 5000 μ g/ml obtained by adding bovine hemoglobin and porcine hemoglobin to the fecal suspension

Each stock solution was diluted by two fold with the fecal suspension to prepare a dilution series. Similarly, enzyme decompositio n products (pepsin treatment) of bovine hemoglobin and porcine hemoglobin corresponding to a hemoglobin concentration of 50 00 μ g/ml were prepared, and a dilution series was prepared for each. Their reactivity with human hemoglobin antibody was me asured with OC Sensor II. The results are shown in Table 2.

[0028]

[Table 2]

ヒト・ヘモグロビンとの交差反応

	牛-ヘモグロビン		豚-ヘモグロビン	
Hb含有量	未処理	酵素分解物	未処理	酵素分解物
(μg/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
20	17	0	67	3
39	35	0	128	2
78	40	0	176	7
156	65	0	244	2
313	106	0	294	4
625	149	2	291	5
1250	173	8	235	11
2500	163	19	157	13
5000	122	34	85	21

[0029]

Due to the prozone phenomenon, the linearity at a measured value of 100ng/ml or more was lost, but about 1 / 1000 of the added amount of bovine hemoglobin showed cross-reactivity with human hemoglobin antibodies, and 1 / 300 of the added amount of porcine hemoglobin showed cross-reactivity. On the other hand, the cross-reactivity of the pepsin-digested hemoglobin degradation product is reduced to 1 / 100,000 or less. It was confirmed that the addition concentration 10-200μg/ml of the hemoglobin decomposition product showing the hemoglobin stabilization effect did not affect the measurement value.

[0030]

Cited document

[1] JP H 5 - 17652Y
[2] JP S 63 - 243756A
[3] JP H 4 - 145366A
[4] JP H 5 - 69466B
[5] JP S 63 - 271160A
[6] JP H 7 - 72154A
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[11] JP H 8 - 29429A
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[15] JP H 8 - 262020A
[16] JP H 2 - 221859A
[17] JP H 7 - 229902A
[18] Japanese Patent Application No. H7-302051

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L1380	140	Louwagie.in. WITH Joost.in.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:39 PM
L1381	802032	Fece$ OR faece$ OR stool	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:39 PM
L1382	36	1380 AND 1381	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:39 PM
L1383	116757940	Process$ OR Collect$	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU,	ADJ	ON	ON	2023/06/28 07:42 PM

			CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L1384	34	1382 AND 1383	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:43 PM
L1385	103575726	divid$ OR partion$ OR portion$ OR separat$	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:45 PM
L1386	473512	1385 AND 1383 AND 1381	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA,	ADJ	ON	ON	2023/06/28 07:45 PM

			VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L1387	64647	1385 SAME 1383 SAME 1381	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:46 PM
L1388	23576	sealable (vessel$ OR container$)	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:48 PM
L1389	17	1385 SAME 1383 SAME 1381 SAME 1388	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:49 PM
L1390	25	1385 SAME 1381 SAME 1388	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB,	ADJ	ON	ON	2023/06/28 07:53 PM

			HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L1391	50544	blood proteins	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:55 PM
L1392	141	blood proteins SAME colorectal cancer	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 07:55 PM
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			IBM_TDB)				
L1394	574594	1381 AND 1383	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 08:03 PM
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L1396	138996	1381 WITH 1383	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 08:03 PM
L1397	277	1381 SAME 1383 AND 1388	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV,	ADJ	ON	ON	2023/06/28 08:04 PM

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L1398	61	1381 SAME 1383 SAME 1388	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 08:04 PM
L1399	11	1381 SAME 1383 SAME 1388 AND 1392	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 08:05 PM
L1400	25	1395 AND 1392	(US-PGPUB; USPAT; USOCR; FIT (AP, AT, AU, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, MC, MD, MY, NL, NO, NZ, OA, PH, PL, PT, RO, RS, RU, SE, SG, SI, SK, SU, TH, TN, TR, TW, UA, VN, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2023/06/28 08:09 PM

PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	1	of	2	*Attorney Docket Number*	EXCTD-35239.307

U.S. PATENTS					
Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		4859610	1989-08-22	MAGGIO	
		5137806	1992-08-11	LEMAISTRE et al.	
		5196167	1993-03-23	GUADAGNO et al.	
		5527676	1996-06-18	VOGELSTEIN et al.	
		5741650	1998-04-21	LAPIDUS et al.	
		5952178	1999-09-14	LAPIDUS et al.	
		11634781	2023-04-25	LOUWAGIE	

U.S. PUBLISHED PATENT APPLICATIONS					
Examiner Initials*	Cite No.[3]	Document Number Number-Kind Code[4] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

Note: Submission of copies of U.S. Patents and published U.S. Patent Applications is not required.

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[5] Number[6] Kind Code[7] *(if known)*	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translation[8]
		EP 1366715	2003-12-03	SENTINEL CH. S.r.L		
		WO 2005/014154	2005-02-17	LOOTS LIMITED		

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]

Examiner Signature		Date Considered	

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	2	of	2	Attorney Docket Number	EXCTD-35239.307

NONPATENT LITERATURE DOCUMENTS			
		AHLQUIST et al., Stool DNA and Occult Blood Testing for Screen Detection of Colorectal Neoplasia. Ann Intern Med. 2008 Oct 7;149(7):441-W81	
		HOEPFFNER et al., Comparative evaluation of a new bedside faecal occult blood test in a prospective multicentre study. Aliment Pharmacol Ther. 2006 Jan 1;23(1):145-54	
		LEUNG et al., Detection of Hypermethylated DNA or Cyclooxygenase-2 Messenger RNA in Fecal Samples of Patients with Colorectal Cancer or Polyps. Am J Gastroenterol 2007;102:1070-6	
		OLSON et al., DNA Stabilization is Critical for Maximizing Performance of Fecal DNA-Based Colorectal Cancer Tests. Diagn Mol Pathol 2005;14:183-191	
		Request for Ex Parte Reexamination of U.S. Patent 11,634,781, filed May 22, 2023, 286 pages	
		Declaration of Mr. Anthony P. Shuber, MS, Reexam Application 90/015,237, filed May 22, 2023, 63 pages	

Examiner Signature		Date Considered	

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	18/179,945
				Filing Date	03/07/2023
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	3	of	2	*Attorney Docket Number*	EXCTD-35239.307

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

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

SIGNATURE

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

Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2023-06-08
Name/Print	Mary Ann D. Brow	Registration Number	42363

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PTO Notes regarding this form:

[1] Applicant's unique citation designation number (optional).
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[5] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3).
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[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**.

EXHIBIT 1005

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(71) Applicant *(for all designated States except US)*: **LOOTS LIMITED**; PO BOX 293, GRANITE HOUSE, LA GRANDE RUE, ST MARTIN GUERNSEY GY1 3RS (GB).

(72) Inventor; and

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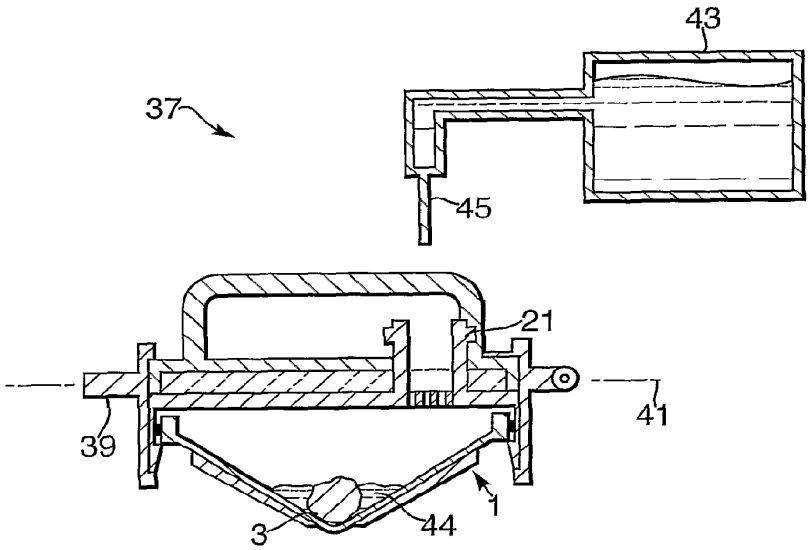
(74) Agents: **GILL, Stephen** et al.; Mewburn Ellis LLP, York House, 23 Kingsway, London Greater London WC2B 6HP (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

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



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

WO 2005/014154 A1

Published:
— *with international search report*

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

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

Field of the Invention

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

Background

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

Exfoliated epithelial cells consist of cellular components, such as DNA, RNA and proteins, which potentially provide a multiplicity of approaches to identifying and monitoring unhealthy conditions of the epithelium as exemplified by colorectal cancer, polyps and ulcerative colitis. The facile recovery of such cells makes possible the application of a wide variety of test procedures for qualitative and/or quantitative analysis of, for example, DNA alterations, gene expression and enzyme activity. A. Loktionov *et al.*, in *Clinical Cancer Research*, Vol. 4, 337-342, (1998), describe a non-invasive screening test for early detection of colorectal cancer which is based on a quantitative comparison of the DNA concentrations derived from exfoliated epithelial cells isolated from the surface of stools from healthy and tumour-bearing persons. In this isolation procedure, cells on the surface of the stool were predominantly clustered and had varying degrees of intactness and damage. In the present specification by "cells" and "cellular material" we mean all such material irrespective of degree of clustering or intactness.

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

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

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

Summary of the Invention

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

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

a) supporting the stool in a container having a channel along which the stool extends;

b) cooling the stool to a temperature below its gel freezing point;

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

If the stool is excreted into the container at home and then transported to e.g. a clinic for washing, the container has the further advantage of holding the stool reasonably securely in the channel so that transportation damage to the stool surface is reduced or avoided.

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

The container may further have installation means (which may be detachable from the container) for removably installing the container on a toilet (e.g. below the toilet seat) with the receiving mouth opening upwardly and the collection channel extending horizontally beneath the mouth. This allows the user to produce the stool in the usual manner, which promotes user acceptability.

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

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

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

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

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

Anaerobic bacteria in stools produce damaging radicals when exposed to atmospheric oxygen (C. F. Babbs (1990) *Free Radical Biology and Medicine*, 8, 191-200). Such oxygen also oxidises the mucus on the surface of stools and encourages the growth of aerobic bacteria. So preferably the container is hermetically sealable to reduce the exposure to atmospheric oxygen of a stool deposited therein. The seal may be achieved by means of a lid or cover (e.g. having a snap-fit or screw fitting) for the mouth. A sealed container rapidly becomes anaerobic due to the high concentration of anaerobic bacteria in the stool, and this apparently largely prevents the growth of aerobic

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

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

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

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

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

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

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

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

Brief Description of the Drawings

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

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

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

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

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

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

Detailed Description

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

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

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

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

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

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

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

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

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

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

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

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

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

When the wash programme ends, the sample rack is pivoted about pivot 47 to bring axis 41 into a vertical position (see Fig. 5). Almost all of wash liquid 44 is then decanted out of receptacle 1 via port 21, foraminous screen 25 substantially preventing larger food residues or other broken off pieces from the stool exiting with the liquid. Coarse sieve 25a removes smaller pieces from the liquid. Finer downstream sieves (not shown) further limit the maximum particle size in the liquid. The liquid is then be divided into standard volumes and received into a plurality of removable (e.g. clip-on) sample receptacles (not shown). The sample receptacles may be a strip of tubes each of e.g. 0.1-0.5 ml volume as can be removed and clipped into a standard microplate format used for clinical analyses. Alternatively the sample receptacles may be individual tubes each of e.g. 1-2 ml volume. The sample rack is then returned to its horizontal position and receptacle 1 is removed and safely disposed of.

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

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

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

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

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

The wash solution was as previously described by A. Loktionov *et al.* (referenced above), but the stool wash was passed through a 250 µm instead of a 125 µm sieve. Cell clean-up was performed robotically on a TECAN™ Genesis RSP 150 and then DNA was released by mechanical cell disruption in the presence of 0.9%SDS as lysing agent. Following dilution to prevent quenching, fluorimetric DNA assay was performed with a TECAN™ Spectrafluor Plus using PicoGreen (Molecular Probes™) to yield the stool DNA index (SDNAI, defined as nanograms of DNA per ml of stool wash).

The effects of pre-soaking, rocking angle, mechanical and bacterial damage, stool age, and wash volume were studied, and the results are given below.

Effect of pre-soaking on SDNAI

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

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

Effect of Rocking Angle

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

Effects of Damage to Stool Surface Layer and of Stool Age

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

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

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

[s] indicates the stool was squashed

[b] indicates the stool had bacterial over-growth

Effect of Wash Solution Volume

Each of a series of undamaged stools from one donor was washed (with a 3 min pre-soak and a 3 min wash at +/- 45° rocking angles) with both a fixed 50 ml volume of wash solution and a variable volume of wash solution based on a 0.5 ml/gram wash/stool ratio. For successive stools of the series, washing first was alternated between the fixed and variable volume. The variable volume based on the 0.5 ratio resulted on average in the use of a 64% greater volume of wash solution which visibly appeared to wet the whole stool better and produced a 21% greater DNA concentration. The second washes gave a 54% higher on average DNA yield than the first washes (largely irrespective of whether the fixed or variable wash solution volume was used first) which was presumably due to the pre-softening effect of the first washes. In general therefore the fixed ratio wash took better account of the stool weight without significantly influencing the DNA result. As shown above, stool age had an insignificant effect on the results.

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

Claims:

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

a) supporting the stool in a container having a channel along which the stool extends;

b) cooling the stool to a temperature below its gel freezing point;

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Fig.1a.

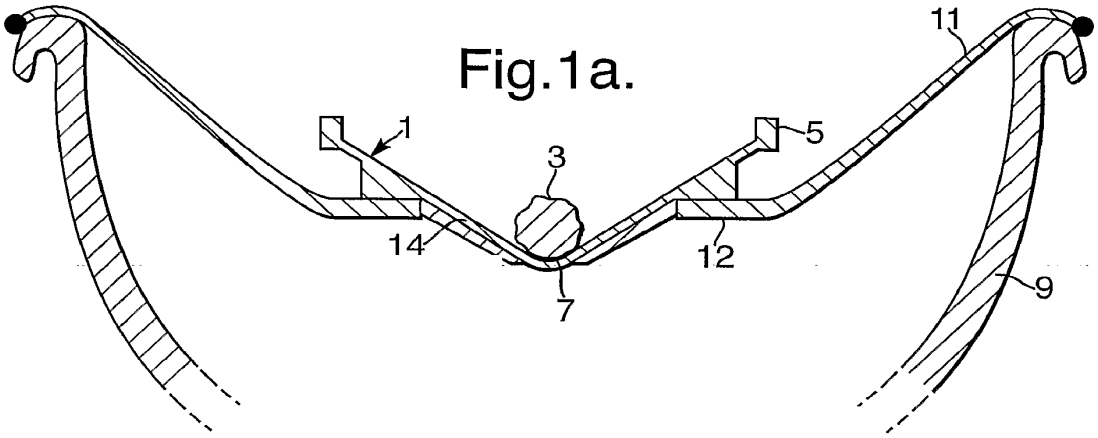


Fig.1b.

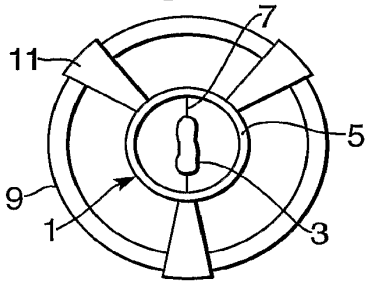


Fig.2.

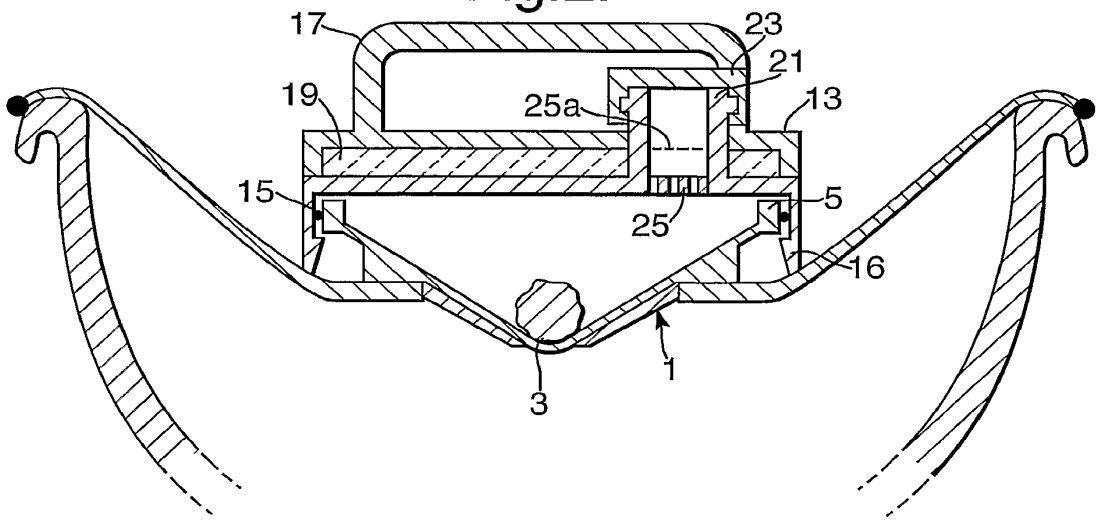


Fig.1c.

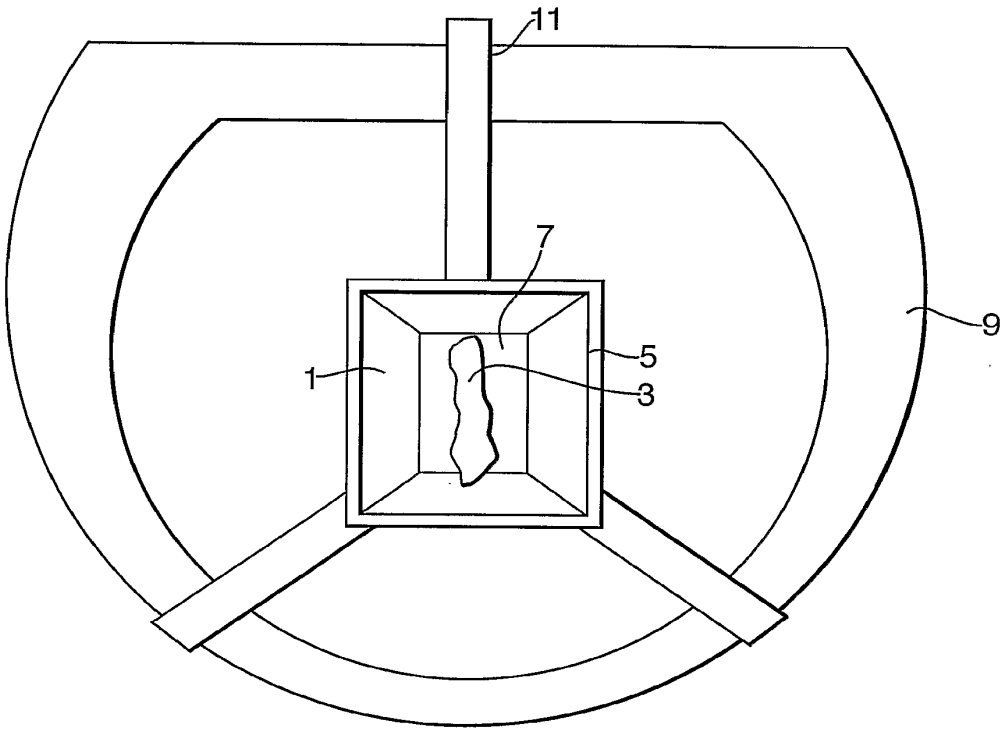


Fig.3a.

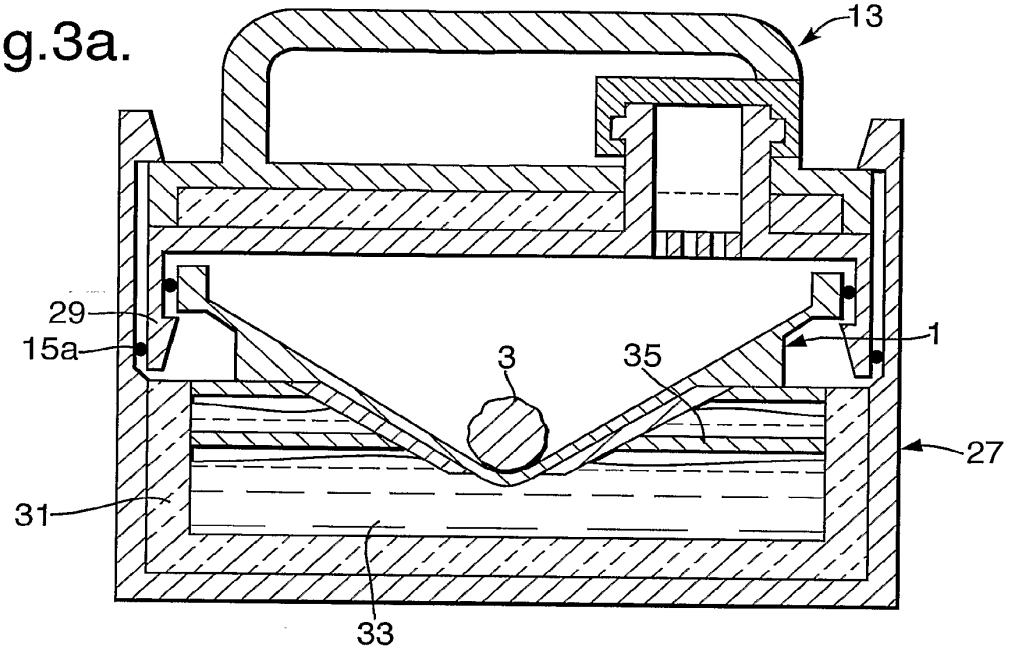

13
29
15a
3
35
1
27
31
33

Fig.3b.

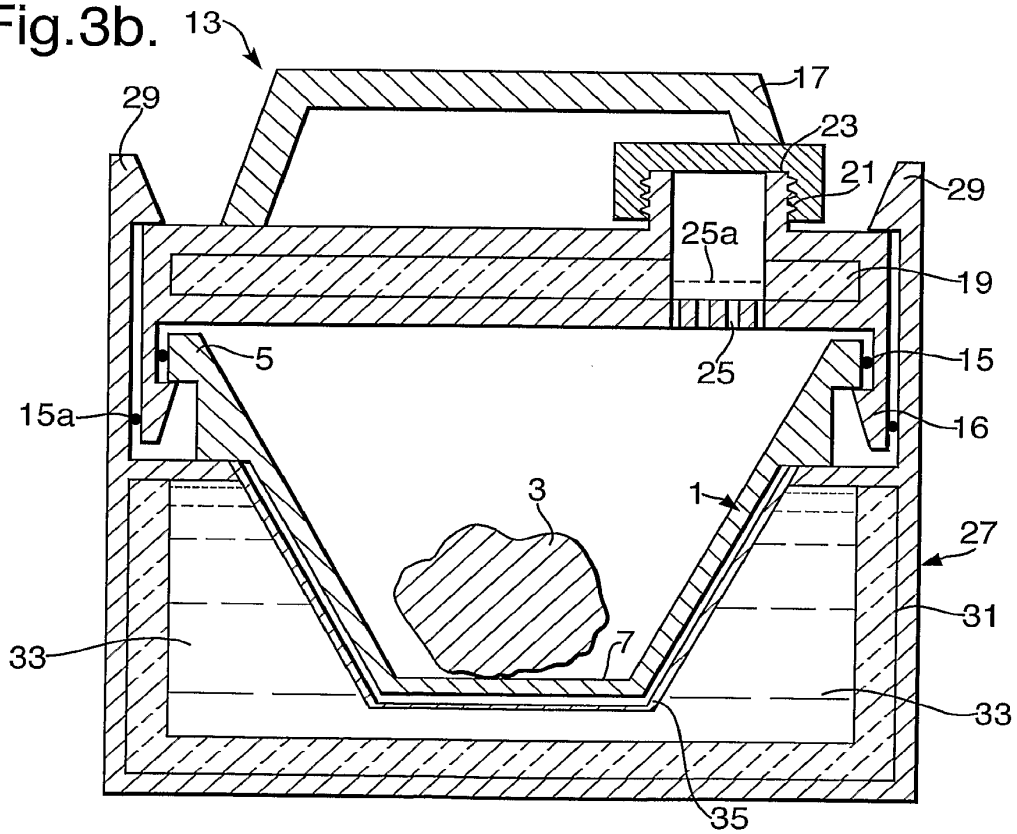

13
17
29
23
21
29
25a
19
5
25
15
15a
16
3
1
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31
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Fig.5.

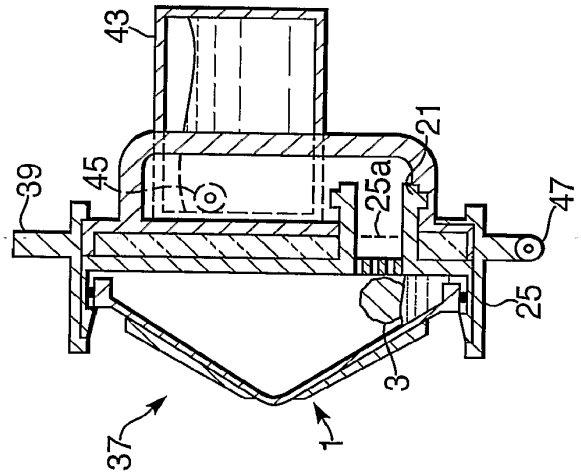
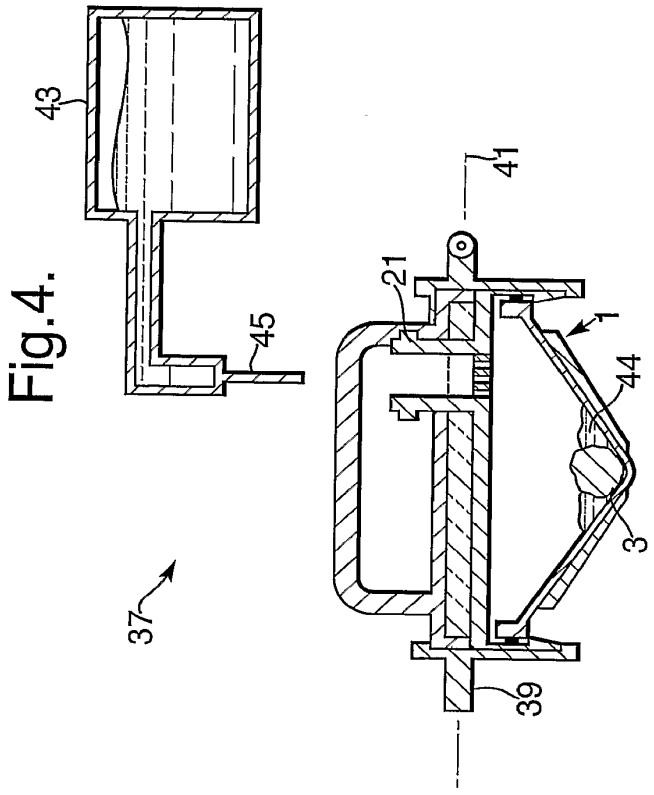


Fig.4.



INTERNATIONAL SEARCH REPORT

nal Application No
B2004/003434

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

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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

-/--

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

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2004	11/11/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Herberhold, C

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INTERNATIONAL SEARCH REPORT

Int[illegible]al Application No
PCT/GB2004/003434

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Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ınal Application No
[illegible]B2004/003434

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APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**06/08/2023 05:13:32 PM ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	62233292	FILING DATE	03/07/2023
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 9

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-06-08_IDS-SB08_EXCTD-003USCON4.35239_35239.pdf	4	Information Disclosure Statement (IDS) Form (SB08)	172 KB
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OLSON_DiagnMolPathol_2005.pdf	9	Non Patent Literature	820 KB
LEUNG_AmJGastro_2007.pdf	8	Non Patent Literature	954 KB

HOEPFFNER_AlimentPharmacolTher_2005.pdf	10	Non Patent Literature	1019 KB
WO2005014154.pdf	31	Foreign Reference	1257 KB
EP1366715.pdf	9	Foreign Reference	480 KB
2023-05-22_Shuber_Decl_11634781.pdf	63	Other reference-Patent/Application/Search Documents	3205 KB
AHLQUIST_AnnInternMed_2008.pdf	23	Non Patent Literature	2103 KB
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European Patent Office
Office européen des brevets

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(51) Int Cl.7: **A61B 10/00**, B01L 3/14, B01L 3/00

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(30) Priority: **31.05.2002 IT MI20020287 U**

(71) Applicant: **Sentinel CH. S.r.L.**
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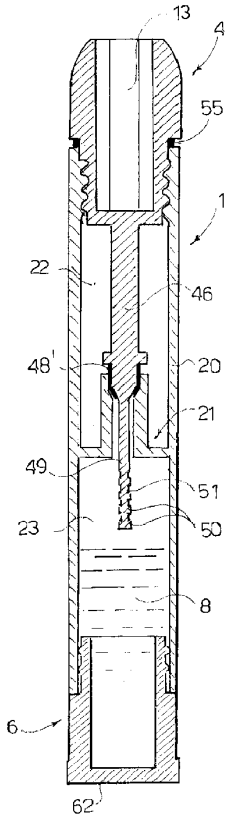
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(54) Extraction tube for collection of faeces samples

(57) An extraction tube (1) is described for the collection of faeces samples, comprising a container body (2) hollow on the inside and open at the top to receive a top cap (4) provided with a threaded small rod (49) for collection of faeces samples. When the top cap (4) is applied to the top end of the container body (2), the threaded small rod protrudes axially into the container body. The container body (2) is also open at the bottom to receive a bottom cap (6). Said extraction tube is employed as a primary collection tube to be inserted in the sample-holder plate of automatic analysers for clinical chemistry and immunological tests.

FIG.3



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EP 1 366 715 A1

Description

[0001] The present invention refers to an extraction and sampling device for collection of faeces samples, particularly for laboratory immunological tests, for qualitative or quantitative determination of one or more analytes, such as haemoglobin, Helicobacter pylori and the like.

[0002] At present, the following two methods, among others, are known for testing for analytes present in faeces samples: the chemical method wherein a defined amount of the sample collected is added to a reagent constituting an active principle which reacts chemically with the analyte under examination giving rise to a chromogenic reaction indicative of the presence of the analyte and the immunological or immunochromographic method wherein the sample collected is dissolved in a buffer solution which is dropped onto a diagnostic device containing an absorbent membrane treated with specific antibodies bound to coloured substances which fix the analytes under examination giving rise to a coloured sign on the membrane.

[0003] These tests are done manually by the operator and are of a qualitative type; that is to say, they reveal the presence or absence of an analyte.

[0004] To carry out sampling of the faeces an extraction tube comprising a cylindrical container with a screw closure cap is used. In the lower inside surface of the cap there is mounted a screw small rod which protrudes axially downwards therefrom. The screw small rod has a threaded portion in its free end, destined for collection of faeces sample. In fact, when the small rod is inserted into the faeces, faeces samples are retained in its thread.

[0005] When the cap is screwed onto the mouth of the container, the screw small rod is partially immersed inside the container, which is suitably filled with a buffer solution having a suitable pH. In this manner the collected faeces sample detaches from the thread of the screw small rod and is dispersed in the buffer solution.

[0006] In the upper outside surface of the cap a breakable tip protruding axially and upward therefrom is provided. Breakage of the tip forms an outlet aperture in the cap communicating with the inside of the container. In this manner the cap acts as a dropper allowing the buffer solution - wherein faeces sample is dissolved - to drop through the outlet aperture.

[0007] During the sampling operation the patient must unscrew the cap from the container, plunge the screw small rod into the faeces so that faeces samples are retained in the thread of the screw small rod, remove with the aid of a cotton wool ball - if possible - the excess faeces accumulated on the small rod outside the thread, and lastly screw the screw cap into the container to immerse the screw small rod in the buffer solution.

[0008] Subsequently, to perform the test, the tip is broken and the buffer solution containing the sample is dropped onto a suitable diagnostic device which contains a nitrocellulose membrane treated with specific antibodies to the analyte (for example haemoglobin) under examination.

[0009] Said collection tube presents some drawbacks. In fact the operation of removing the excess faeces with cotton wool is quite awkward for the user. Furthermore, the outlet aperture of the dropper often becomes obstructed with consequent problems in releasing the solution. Also, the tip of the cap which closes the outlet aperture can break accidentally during handling and/or transport with the result of involuntary release of the solution containing biologically hazardous material.

[0010] The drawback of removal of excess faeces from the screw small rod is solved by known extraction tubes, which provide a dividing partition in the form of an annular flange disposed in the container above the level of the buffer solution. Said dividing flange has a central hole wherein the screw small rod is inserted so that the excess faeces is retained above the dividing flange and does not fall into the buffer solution.

[0011] In this case, the tip of the outlet aperture is disposed in the part of the container opposite the mouth that receives the cap of the screw small rod. Another already known solution provides a device similar to that described above wherein a second screw cap disposed to protect the tip is provided, to prevent accidental breakage thereof.

[0012] However, in extraction tubes of the prior art the problem of occlusion of the outlet aperture of the dropper nevertheless remains. Furthermore said extraction tubes have been designed for manual tests.

[0013] For use in automated systems, the buffer solution containing the dissolved faeces samples must be transferred to special sample-holder tubes which are labelled with identification codes and loaded into an automatic analyser which, in a per se known manner, performs the test.

[0014] It is apparent that said operation of transferring the buffer solution with faeces samples to the sample-holder tube of automatic analysers is quite tiresome for the operator concerned and involves an excessive waste of time.

[0015] It is an object of the present invention to overcome the prior art drawbacks by providing an extraction and sampling tube for the collection of faeces samples that is practical for the patient and simple to use for the laboratory technician.

[0016] Another object of the present invention is to provide an extraction and sampling tube for the collection of faeces samples that is versatile and suitable to be applied directly to different test systems present on the market, such as automatic clinical chemistry and immunological test analysers.

[0017] Yet another object of the present invention is to provide an extraction and sampling tube for faeces collection that is economical and simple to make.

[0018] These objects are achieved according to the invention with the characteristics listed in appended in-

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dependent claim 1.

[0019] Advantageous embodiments of the invention are apparent from the dependent claims. The extraction tube for collection of faeces samples, according to the invention, comprises a container body hollow on the inside for containing a buffer solution and faeces samples, a top cap applied to the top end of the container body and provided with a threaded small rod for sampling of faeces samples and a bottom cap applied removably to the bottom end of the container body.

[0020] The main characteristic of the present invention is that said extraction tube can be used directly for automatic tests.

[0021] In the extraction tube according to the invention the breakable tip provided by the prior art extraction tubes is eliminated and, consequently, all the drawbacks due to the breakable tip are eliminated.

[0022] Furthermore, the upper part of the top cap of the extraction tube according to the invention is advantageously substantially dome-shaped, similarly to the bottom of a primary sampling tube, which makes its use possible on automatic clinical chemistry and immunological test analysers. The diameter of the container body of the extraction device according to the invention is also substantially equal to the diameter of the sample tubes routinely used in laboratories. Consequently the extraction tube according to the invention can be used directly on automatic clinical chemistry and immunological test analysers, without the need of transferring the contents thereof to special test tubes suitable to be positioned in the sample-holder plate of said automatic analysers.

[0023] Further characteristics of the invention will be made clearer by the following detailed description, referring to a purely exemplary and therefore non-limiting embodiment thereof, illustrated in the appended drawings, in which:

Figure 1 is an exploded front view illustrating the extraction tube for collection of faeces samples according to the invention;

Figure 2 is an axial sectional view along the plane of section plane II-II of Figure 1;

Figure 3 is an axial sectional view illustrating the extraction tube according to the invention assembled and with a buffer solution therein;

Figure 4 is an axial sectional view illustrating the extraction tube according to the invention, ready for use in a support of an automatic machine for clinical chemistry and immunological tests, wherein the support is shown broken off.

[0024] The extraction tube for collection of faeces samples according to the invention, designated as a whole with reference numeral 1, is described with the aid of the figures.

[0025] In particular, with reference to Figures 1 and 2 the extraction tube 1 which comprises a container 2, a top cap 4 and a bottom cap 6 is shown exploded.

[0026] The container 2 comprises a substantially cylindrical body 20, hollow on the inside and open at the top and at the bottom. Inside the body 20 of the container, in an intermediate position, a dividing partition 21 is provided which divides the inside of the container 2 into a top chamber 22 and a bottom chamber 23.

[0027] The dividing partition 21 comprises an annular flange 24 which protrudes radially inwards. A cylindrical tang 25 protrudes axially upward from the top of the annular flange 24. The dividing partition 21 has an axial through hole 26 which passes axially through the cylindrical tang 25 and the annular flange 24. The hole 26 has in its upper part a substantially tapered portion 26' with an increasing diameter with respect to the lower part.

[0028] In the inside surface of the container body 20, in the upper end of the top chamber 22, an internal thread 27 is provided.

[0029] The top cap 4 comprises a body consisting of a substantially cylindrical middle part 40 with an outside diameter substantially equal to the outside diameter of the container body 20, a substantially dome-shaped top part 41 disposed above the middle part and a substantially cylindrical bottom part 42, disposed beneath the middle part and having an outside diameter smaller than that of the middle part and slightly smaller than the inside diameter of the container body 20.

[0030] An upwardly open blind hole 43, hexagonal in shape in cross section, is provided axially in the body of the cap 4.

[0031] Knurling 44 consisting of a plurality of longitudinal ribs and grooves is provided in the outside surface of the middle part 40 of the body of the top cap 44.

[0032] An annular seal 55 is provided between the knurled part 44 and the threaded part 45 of the top cap.

[0033] An external thread 45 able to engage, in a screwing relationship, with the internal thread 27 of the container body is provided in the outer surface of the central bottom part 42.

[0034] A stem 46 having a smaller diameter than the bottom part 42 protrudes axially downward from the bottom part 42 of the top cap. At the bottom end of the stem 46 a collar or annular flange 47 which protrudes radially from the stem 46 is provided. The collar 47 has an outside diameter substantially equal to the outside diameter of the tang 25 of the dividing partition of the container 2.

[0035] A tang 48 with a smaller outside diameter than the collar 47 is provided beneath the collar 47. A seal 48' able engage sealably in the tapered part 26' of the hole 26 of the dividing partition 21 is disposed around the tang 48. A small rod 49 with a smaller diameter than the tang 48 and the hole 26 in the dividing partition 21 is provided axially beneath the tang 48.

[0036] A thread formed by a plurality of collars 50 disposed spaced apart from each other so as to form annular spaces 51 between said collars 50 is provided in

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the bottom portion of the small rod 49.

[0037] The bottom cap 6 comprises a body consisting of a middle cylindrical part 60 having an outside diameter substantially equal to the outside diameter of the container 2, a top cylindrical part 61 having a slightly smaller outside diameter with respect to the inside diameter of the container body 20 and a disk-shaped bottom part 62 having an outside diameter slightly greater than the outside diameter of the central part.

[0038] An upwardly open blind hole 63 is provided axially in the body of the bottom cap 6. Knurling 64 consisting of a plurality of longitudinal ribs and grooves is provided in the outer surface of the middle part 60 of the bottom cap 6.

[0039] Annular collars 65 which protrude radially outward so that the top part 61 of the bottom cap can engage by pressure in the bottom part 22 of the container are provided in the outer surface of the top part 61.

[0040] Assembly of the extraction tube 1 is described below with reference to Figure 3. The external thread 45 of the top cap 3 is screwed into the internal thread 27 of the container.

[0041] In this manner the small rod 49 of the top cap 4 enters the axial hole 26 of the dividing partition 21, until the seal 48' of the tang of the small rod settles tightly in the tapered part 26' of the hole 26 in the dividing partition 21 and at the same time the annular seal 55 of the top cap is disposed tightly on the upper edge of the container body 20. In this situation, the threaded part 50 of the small rod 49 is situated inside the bottom chamber 23 of the container, the seal 48' of the tang 48 of the small rod 49 is housed sealably inside the tapered part 26' of the hole 26 of the dividing partition and thus the top chamber 22 of the container is perfectly separated from the bottom chamber 23 of the container.

[0042] At this point a buffer solution 8 is introduced through the bottom opening of the container body 2 and then the bottom cap 6 is applied. The buffer solution 8 can be, for example, a solution with a pH between 7 and 9 with specific stabilizers. It should be noted that, by virtue of the presence of the dividing partition 21 closed by the seal 48' of the tang 48 of the stem of the top cap, the buffer solution 8 cannot pass from the bottom chamber 23 to the top chamber 22 of the container.

[0043] As shown in Figure 3, when the extraction tube 1 is in the upright position, that is to say when the bottom part 62 of the bottom cap 6 rests on a horizontal plane, the buffer solution 8 is partially inside the blind hole 63 of the bottom cap and partially in the bottom chamber 23 of the container, whilst the threaded part 50 of the small rod 49 of the top cap is not immersed in the buffer solution 8.

[0044] The user, when has to collect a faeces sample, unscrews the top cap 4 and plunges the small rod 49 into the faeces. Consequently, on extracting the small rod 49 from the faeces, faeces samples are retained in the annular spaces 51 in the thread 50 of the small rod 49, and also excess faeces remain around the small rod 49.

[0045] Subsequently the user screws the top cap 4 into the container 2. As a result the small rod 49 passes through the hole 26 in the dividing partition and the excess faeces are retained by the upper edge of the tang 25 of the dividing partition and thus remain in the top chamber 22. On the other hand, the faeces required for the analysis is retained in the annular spaces 51 of the thread 50 of the small rod.

[0046] By shaking the extraction tube 1, the buffer solution 8 immerses the threaded part 50 of the screw small rod and, as a result, the faeces samples are dispersed in the buffer solution. At this point the extraction tube 1 can be used as a primary sampling tub to be inserted in the sample-holder plate of automatic clinical chemistry and immunological test analysers.

[0047] As shown in Figure 4, in the extraction tube 1, in the upturned position, the buffer solution 8 occupies the bottom chamber 23 and totally immerses the threaded part 50 of the small rod 49. It should be noted that, even in this situation, due to the presence of the dividing partition 21 which is closed by the seal 48' of the tang of the small rod, the buffer solution 8 cannot pass from the bottom chamber 23 to the top chamber 22 of the container.

[0048] At this point the extraction tube 1 is positioned in a special housing 9 of an automatic machine for immunological tests and then the bottom cap 6 is removed so as to allow removal of the buffer solution 8 containing the faeces samples by a probe of the automatic machine.

[0049] It should be noted that the dome shape 41 of the upper part of the top cap 4 is designed to be substantially similar to the bottom of test tubes typically used in automatic analysers for clinical chemistry and immunological tests, to allow insertion of the extraction tube 1 inside the housing 9 of the automatic test machine.

[0050] Numerous changes and variations of detail within the reach of a person skilled in the art can be made to the present embodiment of the invention, without thereby departing from the scope of the invention as set forth in the appended claims.

Claims

1. An extraction tube (1) for collection of faeces samples comprising:

   - a container body (2), hollow on the inside, open at the top, and able to receive a buffer solution,
   - a top cap (4) provided with a threaded small rod (49) for collection of faeces samples, said threaded small rod protruding axially inside the container body, when the top cap (4) is applied to the top end of the container body (2),
   - a dividing partition (21) provided, in an interme-

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diate position, inside said container body (2) so as to separate a top chamber (22) from a bottom chamber (23) inside said container body, said dividing partition (21) having an axial hole (26) suitable to allow the passage of said threaded small rod (49), so as to retain the excess faeces in said top chamber (22) and allow the passage of the threaded part of the small rod into said bottom chamber (23),

characterised in that said container body is open at the bottom and provided with a bottom cap (6) which can be applied movably to the bottom end of the container body, so that said extraction tube can be used directly as a primary sampling tube to be inserted in a sample-holder plate of automatic analysers, following removal of said bottom cap (6) and overturning of said container body (2) for positioning in a housing (9) of the automatic analyser, with the top cap (4) facing downward.

2. An extraction tube (1) according to claim 1, **characterised in that** said top cap (4) has an upper portion (41) destined to be disposed outside said container body (2), said upper portion (41) having a substantially domed shape similar to the bottom of a test tube normally used in the sample-holder plate of automatic analysers.

3. An extraction tube according to claim 1 or 2, **characterised in that** said top cap (4) comprises a stem (46) ending in an annular flange (47) beneath which is provided a cylindrical tang (48) surrounded by a seal (48') beneath which said threaded small rod (49) protrudes axially, and said dividing partition (21) comprising an annular flange (24) from which a cylindrical tang (25) protrudes axially upward so that when said top cap (4) is applied to the top end of the container body, the seal 48' of the cylindrical tang (48) of the stem (46) of the top cap is housed sealably inside a tapered part (26') of said hole (26) in the dividing partition.

4. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said top cap (4) comprises a bottom portion (42) having an external thread (45) able to engage in an internal thread (27) provided in the top inner part of said container body (2).

5. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said top cap (4) comprises a middle portion (40) having knurling (42) consisting of a plurality of longitudinal ribs and grooves.

6. An extraction tube (1) according to claims 4 and 5, **characterised in that** between said knurled part (44) of the top cap and said threaded part (45) of the top cap there is provided an annular seal (55) able to abut sealably on the top edge of said container body 2.

7. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said top cap (4) has a blind hole (43) axially open at the top to the outside of the container body, and hexagonal in shape in cross section.

8. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said bottom cap (6) has a top portion (61) with a plurality of annular collars (65) protruding radially outwards, to be able to be applied by pressure into the bottom part of said container body (2).

9. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said bottom cap (6) comprises a middle portion (60) with knurling (64) consisting of a plurality of longitudinal ribs and grooves.

10. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said bottom cap (6) has a blind hole (63) axially open at the top to the inside of the container body (2).

11. An extraction tube (1) according to any one of the preceding claims, **characterised in that** said threaded small rod (49) has in its bottom portion a thread consisting of a plurality of concentric collars (50) so as to define a plurality of annular spaces (51) therebetween, able to retain the sampled faeces.

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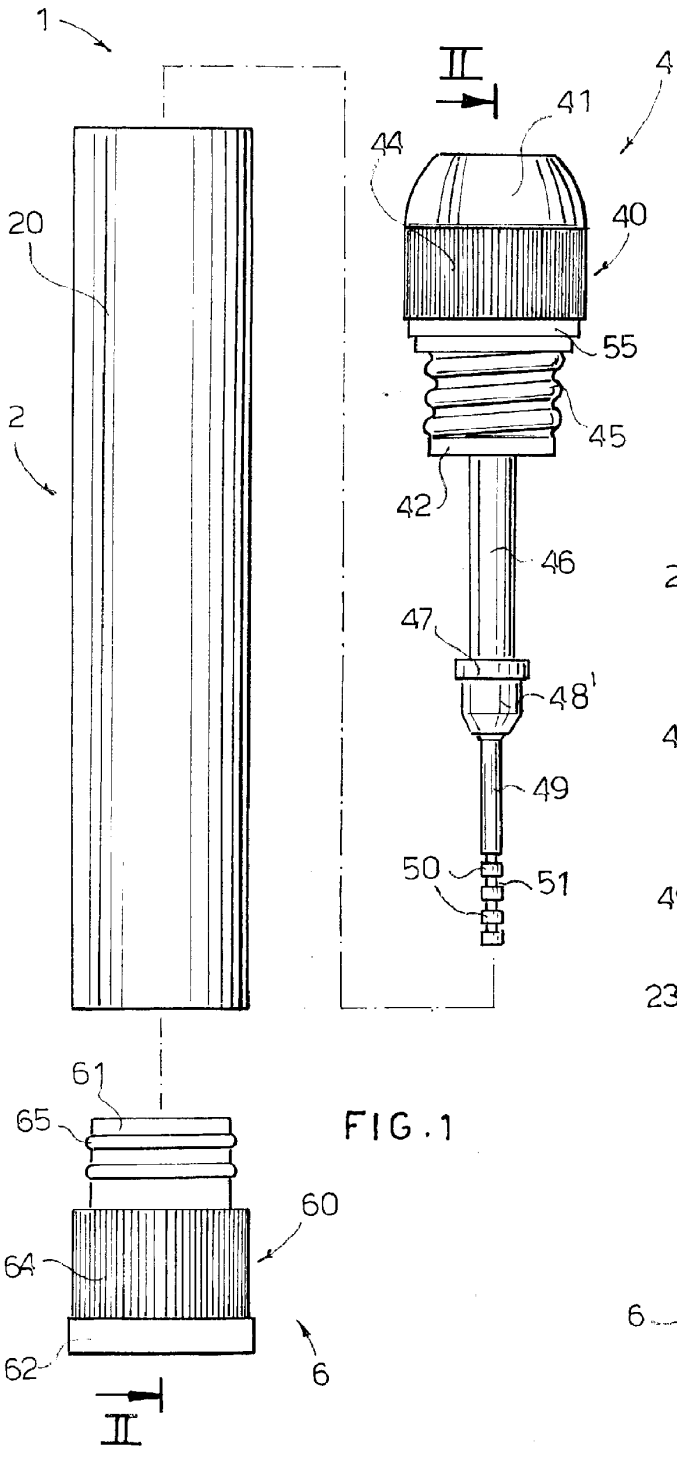

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FIG. 1

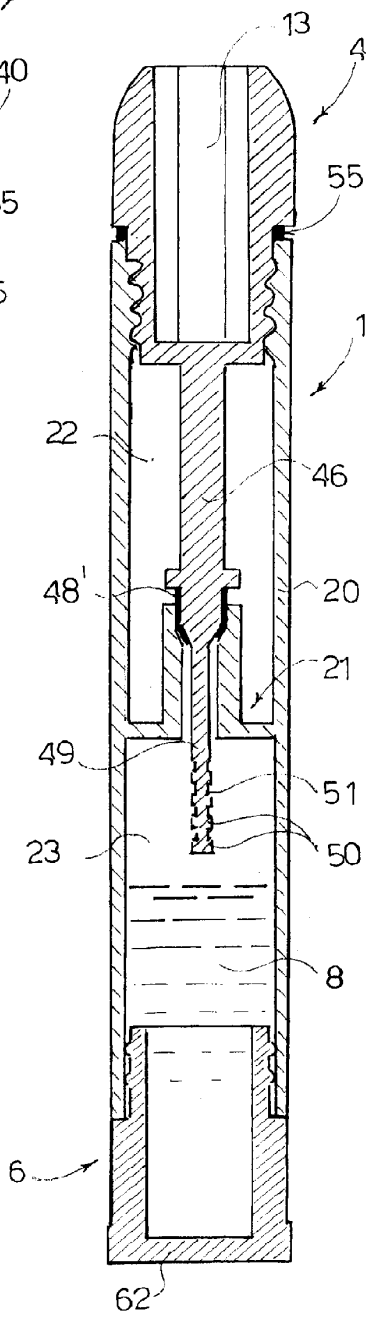

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FIG. 3

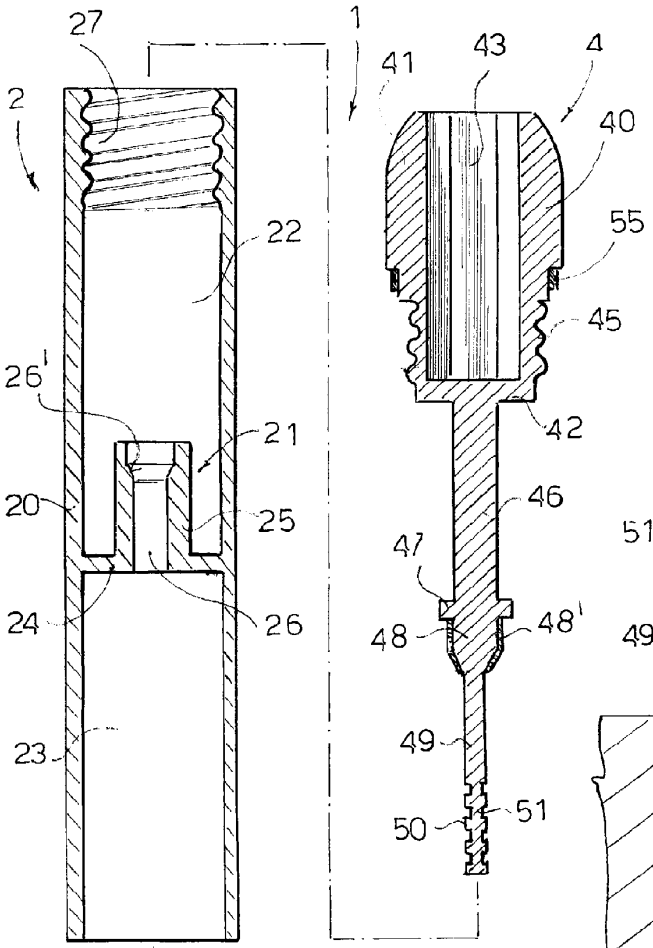

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FIG. 2

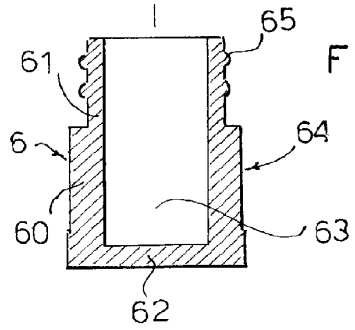
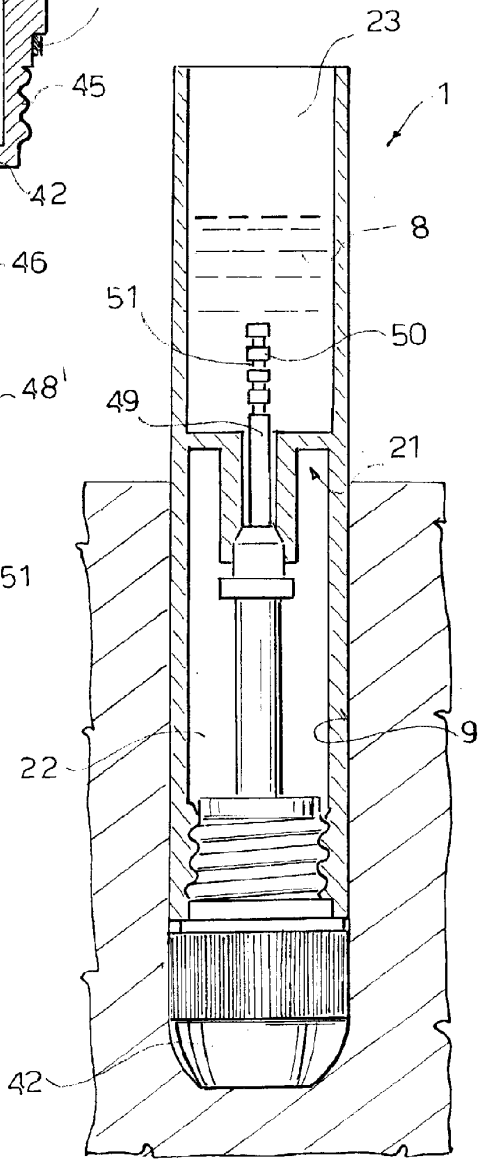

65
61
6
64
60
63
62

FIG. 4


23
1
8
51
50
49
21
9
22
42

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 03 01 1896

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	EP 0 727 653 A (WAKO PURE CHEM IND LTD) 21 August 1996 (1996-08-21) * column 3, line 17 - line 38 * * column 4, line 58 - column 5, line 6 * * figure 1 * ---	1	A61B10/00 B01L3/14 B01L3/00
A	US 6 299 842 B1 (KOZAK KENNETH JAMES ET AL) 9 October 2001 (2001-10-09) * column 3, line 53 - column 4, line 13 * * figure 1 * -----	1	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

A61B
B01L
G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 July 2003	Compos, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 03 01 1896

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

21-07-2003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0727653 A	21-08-1996	DE 69610569 D1	16-11-2000
		DE 69610569 T2	31-05-2001
		EP 0727653 A2	21-08-1996
		ES 2151090 T3	16-12-2000
		JP 8285845 A	01-11-1996
		US 6207113 B1	27-03-2001
		US 5882942 A	16-03-1999
US 6299842 B1	09-10-2001	AU 3727200 A	21-09-2000
		WO 0051496 A1	08-09-2000
		US 2002007686 A1	24-01-2002

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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

173254 7590 06/05/2023

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

EXAMINER
CENTRAL, DOCKET

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
06/05/2023	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

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 18/179,945	**Applicant(s)** Louwagie, Joost	
	Examiner KIMBERLY A INABINET	**Art Unit** OPET	**AIA (FITF) Status** No

1. THE REQUEST FILED 07 March 2023 IS **GRANTED** .

The above-identified application has met the requirements for prioritized examination

A. ☑ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination**. The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;

B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims** , or a multiple dependent claim;

C. filing a **request for continued examination** ;

D. filing a notice of appeal;

E. filing a request for suspension of action;

F. mailing of a notice of allowance;

G. mailing of a final Office action;

H. completion of examination as defined in 37 CFR 41.102; or

I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to KIMBERLY INABINET at (571) 272-4618. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/KIMBERLY A INABINET/ Paralegal Specialist, OPET	

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
18/179,945	03/07/2023		1820	EXCTD-35239.307	20	1

CONFIRMATION NO. 2966

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

FILING RECEIPT

OC000000057308692

Date Mailed: 05/22/2023

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

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

Inventor(s)

Joost Louwagie, Dornach, SWITZERLAND;

Applicant(s)

Exact Sciences Corporation, Madison, WI;

Power of Attorney: The patent practitioners associated with Customer Number 173254

Domestic Priority data as claimed by applicant

This application is a CON of 17/936,335 09/28/2022 PAT 11,634,781
which is a CON of 15/634,607 06/27/2017
which is a CON of 15/010,436 01/29/2016 ABN *
which is a CON of 13/147,570 03/12/2012 ABN
which is a 371 of PCT/GB2010/000180 02/03/2010
which claims benefit of 61/149,581 02/03/2009

(*) Data provided by applicant is not consistent with PTO records.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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

If Required, Foreign Filing License Granted: 05/19/2023

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 18/179,945**

Projected Publication Date: 08/31/2023

Non-Publication Request: No

Early Publication Request: No

Title

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Preliminary Class

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

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

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

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

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

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

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 18/179,945

APPLICATION AS FILED - PART I

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

APPLICATION AS AMENDED - PART II

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

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

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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

Application Number	Filing Date
18/179,945	07-Mar-2023

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SIGNATURE of Applicant for Patent

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Signature	/Jennifer M. Duke/	Date (Optional)	
Name	Jennifer M. Duke		
Title	Sr. Director, Lead Counsel Intellectual Property		

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

☑ Total of 1 forms are submitted.

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

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

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

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**04/20/2023 03:43:32 PM ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Jeremiah Gile
PATENT CENTER #	61957225	FILING DATE	-
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
2023-03-07_35239-307_ExecPOA.pdf	2	Power of Attorney	162 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized

by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

Application Number: 18179945

Reviewed by: kBuLmOjFP3VzlESORWEnm76I/npXqkJwHo0JQ1qYXov9ZBL4CQRFMQ==

Note: This validation report concerns the sequence listing filed 03-07-2023 18:07:59 and indicates that NO errors were found by the USPTO during validation of the sequence listing. This validation report should not be construed as meaning that the sequence listing complies with all of the requirements of 37 CFR §§ 1.821-1.825, 37 CFR §§ 1.831-1.835 and 1.839, WIPO Standard ST.25, or WIPO Standard ST.26.

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

Timestamp: [year=2023; month=3; day=8; hr=8; min=34; sec=47; ms=191]

Validated By CRFValidator v 1.5.0

Application No: 18179945 Version No: 1.1

CRF Copy Request from application Version :

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Elapsed: 0 hr(s) 0 min(s) 11 sec(s) 530 ms

Total Warnings: 1

Total Errors: 0

No. of SeqIDs Defined: 39

Actual SeqID Count: 39

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	1	of	5	Attorney Docket Number	EXCTD-35239.307

U.S. PATENTS					
Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		4437975	1984-03-20	GILLESPIE et al.	
		4445235	1984-05-01	SLOVER et al.	
		4582811	1986-04-15	PUCCI et al.	
		4683197	1987-07-28	GALLATI	
		4889818	1989-12-26	GELFAND et al.	
		5198365	1993-03-30	GROW et al.	
		6410276	2002-06-25	BURG et al.	
		6761702	2004-07-13	SMITH	
		7288413	2007-10-30	GOULDEN	
		7371527	2008-05-13	BAYLIN et al.	
		8969046	2015-05-03	VAN ENGELAND et al.	

U.S. PUBLISHED PATENT APPLICATIONS					
Examiner Initials*	Cite No.[3]	Document Number Number-Kind Code[4] *(if known)*	Issue or Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20020187476	2002-12-12	KOROULIS et al.	
		20020096469	2002-07-25	FAULKNER	
		20030086869	2003-05-08	STALLINGS	
		20030096244	2003-05-22	RABELLO et al.	
		20040019298	2004-01-29	ZHOU et al.	
		20040091881	2004-05-13	OLEK et al.	
		20050064401	2005-03-24	OLEK et al.	
		20050075543	2005-04-07	CALABRESE	
		20050244836	2005-11-03	TSANG	
		20060084054	2006-04-20	ALSOBROOK et al.	
		20060188939	2006-08-24	GAO	
		20060210448	2006-09-21	WANG et al.	

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	2	of	5	Attorney Docket Number	EXCTD-35239.307

U.S. PUBLISHED PATENT APPLICATIONS					
		20060216830	2006-09-28	KIKUIRI	
		20070017015	2007-01-25	FINELL	
		20070072214	2007-03-29	GARVIN	
		20080097238	2008-04-24	LOKTIONOV et al.	
		20080124714	2008-05-29	SHUBER et al.	
		20080221056	2008-09-11	BAYLIN et al.	
		20080227208	2008-09-18	YEE et al.	
		20090004058	2009-01-01	LIANG et al.	
		20100136572	2010-06-03	ATAMAN-ONAL et al.	
		20120164238	2012-06-28	LOUWAGIE	
		20160194723	2016-07-07	LOUWAGIE	
		20230048152	2023-02-16	LOUWAGIE	

Note: Submission of copies of U.S. Patents and published U.S. Patent Applications is not required.

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[5] Number[6] Kind Code[7] (*if known*)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translation[8]
		EP 0032782	1981-07-29	KOMMANDIITTIYHTIO FINNPIPETTE		
		EP 0308227	1989-03-22	SMITHKLINE DIAGNOSTICS, INC.		
		EP 0817968	1998-01-14	EXACT SCIENCES CORPORATION		
		FR 2919065	2009-01-23	BIOMERIEUX		
		WO 1990/06995	1990-06-28	SISKA DIAGNOSITCS, INC.		
		WO 1997/46705	1997-12-11	THE JOHNS HOPKINS SCHOOL OF MEDICINE		
		WO 2000/050640	2000-08-31	EXACT LABORATORIES, INC.		
		WO 2004/067726	2004-08-12	KECK GRADUATE INSTITUTE		

Examiner Signature		Date Considered	

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	3	of	5	Attorney Docket Number	EXCTD-35239.307

FOREIGN PATENT DOCUMENTS						
		WO 2004/083399	2004-09-30	THE JOHNS HOPKINS UNIVERSITY.		
		WO 2004/0927209	2004-10-28	KIETURAKIS & KARCZEWSKI		
		WO 2005/014154	2005-02-17	LOOTS LIMITED		
		WO 2005/017207	2005-02-24	CASE WESTERN RESERVE UNIVERSITY		
		WO 2006/113671	2006-10-26	ONCOMETHYLOME SCIENCES SA		
		WO 2008/010975	2008-01-24	THE JOHNS HOPKINS UNIVERSITY		
		WO 2008/084219	2008-07-17	ONCOMETHYLOME SCIENCES SA		
		WO 2008/100913	2008-08-21	THE JOHNS HOPKINS UNIVERSITY		
		WO 2008/102002	2008-08-28	RIKSHOSPITALET RADIUMHOSPITALE		

NONPATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and-or country where published.	Translation[6]
		ABBASZADEGAN et al., Stool-based DNA testing, a new noninvasive method for colorectal cancer screening, the first report from Iran. World J Gastroenterol. 2007 Mar 14;13(10):1528-33	
		AHLQUIST et al., Cologuard Primed to Change Landscape of CRC Screenin", Mayo Clinic Clinical Updates, http://www.mayoclinic.org/medical-professionals/clinical-updates/digestivediseases/ cologuard-primed-to-change-landscape-of-crc-screening, Pages 1-4, 12/03/2014.	
		AUERKARI, Methylation of tumor suppressor genes p16(INK4a), p27(Kip1) and E-cadherin in carcinogenesis. Oral Oncol. 2006 Jan;42(1):5-13	
		CHEN et al., Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst. 2005 Aug 3;97(15):1124-32	
		CONROY et al., Exact Sciences Completes 40,000 Cologuard Tests During First Quarter 2016, Exact Sciences Latest News, Pages 1-10, 05/03/2016.	

Examiner Signature		Date Considered	

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	4	of	5	Attorney Docket Number	EXCTD-35239.307

NONPATENT LITERATURE DOCUMENTS			
		EXACT SCIENCES, Cologuard(TM) sDNA-based Colorectal Cancer Screening Test - Instructions for Use, Pages 1-71, 2013.	
		HAMMER, Human hybirds. Sci Am. 2013 May;308(5):66-71	
		ITZKOWITZ et al., A simplified, noninvasive stool DNA test for colorectal cancer detection. Am J Gastroenterol. 2008 Nov;103(11):2862-70	
		KARL et al., Improved diagnosis of colorectal cancer using a combination of fecal occult blood and novel fecal protein markers. Clin Gastroenterol Hepatol. 2008 Oct;6(10):1122-8	
		KIM et al., Noninvasive Molecular Biomarkers for the Detection of Colorectal Cancer. BMB Rep. 2008 Oct 31;41(10):685-92	
		KUTZNER et al., Non-invasive detection of colorectal tumours by the combined application of molecular diagnosis and the faecal occult blood test. Cancer Lett. 2005 Nov 8;229(1):33-41	
		LAIRD, The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003 Apr;3(4):253-66	
		LENHARD et al., Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. Clin Gastroenterol Hepatol. 2005 Feb;3(2):142-9	
		LEVI et al., A quantitative immunochemical faecal occult blood test is more efficient for detecting significant colorectal neoplasia than a sensitive guaiac test. Aliment Pharmacol Ther. 2006 May 1;23(9):1359-64	
		OHLSSON et al., Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR. Br J Cancer. 2006 Jul 17;95(2):218-25	
		MULDER et al., Tumor pyruvate kinase isoenzyme type M2 and immunochemical fecal occult blood test: performance in screening for colorectal cancer. Eur J Gastroenterol Hepatol. 2007 Oct;19(10):878-82	
		REIN et al., Identifying 5-methylcytosine and related modifications in DNA genomes. Nucleic Acids Res. 1998 May 15;26(10):2255-64	
		SHASTRI et al., Comparison of an established simple office-based immunological FOBT with fecal tumor pyruvate kinase type M2 (M2-PK) for colorectal cancer screening: prospective multicenter study. Am J Gastroenterol. 2008 Jun;103(6):1496-504	
		SOMMER et al., Minimal homology requirements for PCR primers. Nucleic Acids Res. 1989 Aug 25;17(16):6749	
		TURGEON et al., Fecal DNA-Based Detection of Colorectal Neoplasia. Curr Colorectal Cancer Rep. 2007 Oct;3(4):171-177	

Examiner Signature		Date Considered	

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
				Application Number	
				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	5	of	5	Attorney Docket Number	EXCTD-35239.307

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

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)				Complete if Known	
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				Filing Date	
				First Named Inventor	JOOST LOUWAGIE
				Art Unit	
				Examiner Name	
Sheet	6	of	5	Attorney Docket Number	EXCTD-35239.307

CERTIFICATION STATEMENT

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

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

OR

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

☐ See attached certification statement.

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

☒ A certification statement is not submitted herewith.

☒ Documents not provided herewith have been previously cited in parent U.S. patent application number 13/147,570 filed on March 12, 2012; 15/010,436, filed on January 29, 2016; 15/634,607 filed on June 27, 2017; and/or 17/936,335 filed on September 28, 2022. In compliance with 37 C.F.R. § 1.98(d), Applicants have not included copies of these documents.

SIGNATURE

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

Signature	/Mary Ann D. Brow/	Date (YYYY-MM-DD)	2023-03-07
Name/Print	Mary Ann D. Brow	Registration Number	42363

.

PTO Notes regarding this form:

[1] Applicant's unique citation designation number (optional).
[2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04.

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

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	03/07/2023 06:07:59 PM ET	EXCTD-35239.307

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	61703441	AUTHORIZED BY	Mary Ann Brow
CUSTOMER #	173254	FILING DATE	-
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Joost Louwagie

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0638	E202337I08235000	Stephanie Filandrinos

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

TOTAL AMOUNT:	$6,160.00

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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

Title of Invention	Methods of Detecting Colorectal Cancer

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 15/010,436 filed on 29-Jan-2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

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

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Joost Louwagie Date (Optional): 23 Jan 2016

Signature:

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

Application Data Sheet 37 CFR 1.76

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

Inventor Information

of inventors: 1

1. **Joost Louwagie**

Residence Information
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Application Information

Customer number	**173254 -**
Correspondence address	**---**
Title of invention	**FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD**
Attorney docket number	**EXCTD-35239.307**
Entity status	Regular Undiscounted
Application type	Nonprovisional Application under 35 USC 111(a)

Subject matter	Utility
Total number of drawing sheets	---
Suggested figure for publication	---
Filing by reference	No
Publication request	Normal eighteen-month publication

Representative Information

of representatives: 1

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

Customer number 173254

Domestic Benefit/National Stage Information

of benefit claims: 6

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

Prior app status	Pending
Application number	---
Continuity type	**Continuation of**
Prior app number	**17936335**
Filing Date	09/28/2022

Prior app status	Pending
Application number	**17936335**
Continuity type	**Continuation of**

Prior app number	**15634607**
Filing Date	06/27/2017

Prior app status	Abandoned
Application number	**15634607**
Continuity type	**Continuation of**
Prior app number	**15010436**
Filing Date	01/26/2016

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

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

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

Foreign Priority Information

of foreign priority claims: 1

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the Application Data Sheet constitutes the claim for priority as required by 35 U.S.C. 119 (b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(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).

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

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

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

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013. NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 2016, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization or Opt-Out of Authorization to Permit Access

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

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

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

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

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 the search results from the instant application.

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

Applicant Information

of applicants: 1

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

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

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

Applicant Organization
Applicant type Assignee

Assignee Information including Non-Applicant Assignee Information

of assignees: 0

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

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

Data was not provided for this section.

Signature

NOTE:

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

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

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

Signature	First name	Last name	Registration #	Date
/Mary Ann D. Brow/	Mary Ann D.	Brow	42363	03/07/2023

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/179,945	**03/07/2023 06:07:59 PM ET**	**EXCTD-35239.307**

Title of Invention

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2966	FILED BY	Stephanie Filandrinos
PATENT CENTER #	61703441	FILING DATE	-
CUSTOMER #	173254	FIRST NAMED INVENTOR	Joost Louwagie
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Mary Ann Brow

Documents

TOTAL DOCUMENTS: 8

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
generatedADS61703441.pdf	6	Application Data Sheet	138 KB
2023-03-07_35239-307_TrackOne_Form.pdf	2	Track One Request	117 KB
35239US4CON_Exec_Decl.pdf	1	Oath or Declaration filed	106 KB
Warning: The attached file contains comments or annotations. Comments are not recommended and may cause processing problems with the document.			
35239-307_SEQUENCE_LISTING.xml	-	Sequence Listing (ST.26 XML File)	35 KB

2023-03-07_APPLICATION_EXCTD-003USCON4.35239_35239.pdf		62	-	577 KB
2023-03-07_APPLICATION_EXCTD-003USCON4.35239_35239-SPEC.pdf	(1-57)	57	Specification	569 KB
2023-03-07_APPLICATION_EXCTD-003USCON4.35239_35239-CLM.pdf	(58-61)	4	Claims	140 KB
2023-03-07_APPLICATION_EXCTD-003USCON4.35239_35239-ABST.pdf	(62-62)	1	Abstract	107 KB
2023-03-07_35239-307_IDS-SB082.pdf		7	Information Disclosure Statement (IDS) Form (SB08)	196 KB

Warning: This is not a USPTO supplied IDS fillable form. Data in the form cannot be automatically loaded to other USPTO systems.

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
generatedADS61703441.pdf	BD4C841F514981C84CF1231FFE6621D842B0FAB4C8F090ED7A8CF0ACC39FBFEA07BAB6EE7FC6CD9BF42118456751 2B81E5E1852677D8D400FEAE0B8783974A1A
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35239US4CON_Exec_Decl.pdf	F4E816446026739CF274E7C2E136426C9CE270A41A10F6E5AE7EECE308CFC4C0A0E0C3F80970E8D5569641BB10D813E48137F8E5E3996471B0BEB6A00ACF242B
35239-307_SEQUENCE_LISTING.xml	BE36D9B80DA426E67C22DE34D81E202F34902C27300796EDD7CD5C386CBD5D04FAF5007364D132418581AEEE256817F0170B20C31032590A3D82E4487C14541D
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2023-03-07_APPLICATION_EXCTD-003USCON4.35239_35239-ABST.pdf	841C19C36BCCE2CB87706ED81C60149A995706B13BD0D61C5631A02D981A83291624521851ADDFBC8A65D4F097A67E2C52EF3B017A1A325A94B45AEBF250A38C
2023-03-07_35239-307_IDS-SB082.pdf	7DBF62F47EAC71D962CCC2DA9D56CE0CC3BC8C6E4A029B7FA297DDC78629A6F071C12C7098FBDA88901CA95905816E7FCF99956B4B35B12C3ED033DB1A064C13

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

New Applications Under 35 U.S.C. 111

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

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

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage

submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

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

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

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

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

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

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

I. ☑ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. ☐ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Mary Ann D. Brow/	Date 2023-03-07
Name (Print/Typed) Mary Ann D. Brow	Practitioner Registration Number 42363

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

☑ *Total of 1 forms are submitted.

Privacy Act Statement

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

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

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

FECAL SAMPLE PROCESSING AND ANALYSIS COMPRISING DETECTION OF BLOOD

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application No. 17/936,335, filed September 28, 2022, now allowed, which is a continuation of U.S. Patent Application No. 15/634,607, filed June 27, 2017, which is a continuation of U.S. Application No. 15/010,436, filed January 29, 2016, now abandoned, which is a continuation of U.S. Application No. 13/147,570, filed March 12, 2012, now abandoned, which is the U.S. national stage of International Application PCT/GB2010/000180, filed February 3, 2010, which claims priority to U.S. Provisional Application No. 61/149,581, filed February 3, 2009. The contents of these applications are incorporated herein by reference in their entireties.

SEQUENCE LISTING

The text of the computer readable sequence listing filed herewith, titled "35239-307_SEQUENCE_LISTING", created March 6, 2023, having a file size of 35,640 bytes, is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide, and is the second leading cause of cancer-related deaths in the United States. A patient's prognosis is good if the cancer is caught early, when the site of the cancer is confined to its site of

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

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

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

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

Fecal occult blood tests fall primarily into two categories, tests based on the use of chromogenic chemical reagents such as gum guaiac and immunochemical tests. The chemically based guaiac methods determine the presence of occult blood by the detection of the perioxidase activity of the hemoglobin in the blood present in the faecal sample. They require catalysis of peroxide into oxygen and water, and the subsequent oxidation of a

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

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

designed to detect long DNA fragments, which have been specifically associated with cells called non-apoptotic colonocytes, which are common in CRC. While this test is more sensitive than fecal occult blood testing, it is not as sensitive as colonoscopy and will miss about half of cancers in an average risk group of people without symptoms.

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

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

Although combined assays for detecting CRC have been described, their approach targets either multiple protein markers or either multiple DNA alterations. To date, immunochemical tests and DNA tests for CRC detection have been evaluated and compared on a separate basis only.

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

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

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

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

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

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

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

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

SUMMARY OF THE INVENTION

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

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

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

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

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

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

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

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

In related aspects, the invention provides

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Also provided is a kit for any of:

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

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

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

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

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

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

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

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

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

(d) selecting a suitable treatment regimen for colorectal cancer comprising means for detecting an epigenetic modification in at least one gene selected from LAM A1 and CD01.

The invention also provides a kit for any of:

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

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