(12) STANDARD PATENT (11) Application No. AU 2016277943 B2
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Integrated purification and measurement of DNA methylation and co-measurement of mutations and/or mRNA expression levels in an automated reaction cartridge

(51) International Patent Classification(s)
B01L 3/00 (2006.01) ***C12Q 1/68*** (2006.01)
C12N 15/10 (2006.01)

(21) Application No: **2016277943** (22) Date of Filing: **2016.06.14**

(87) WIPO No: **WO16/205233**

(30) Priority Data

(31) Number	(32) Date	(33) Country
62/175,916	**2015.06.15**	**US**

(43) Publication Date: **2016.12.22**
(44) Accepted Journal Date: **2022.09.01**

(71) Applicant(s)
Cepheid

(72) Inventor(s)
Lai, Edwin W.;Kohlway, Andrew;Vanatta, Reuel;Higuchi, Russell;Gall, Alexander;Kocmond, Kriszten

(74) Agent / Attorney
Pizzeys Patent and Trade Mark Attorneys Pty Ltd, GPO Box 1374, Brisbane, QLD, 4001, AU

(56) Related Art
WO 00/73412 A2
WO 2014/052551 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) **World Intellectual Property Organization**
International Bureau

(43) **International Publication Date**
22 December 2016 (22.12.2016)

WIPO | PCT

(10) **International Publication Number**
WO 2016/205233 A3

(51) **International Patent Classification**:
C12N 15/10 (2006.01) *B01L 3/00* (2006.01) *C12Q 1/68* (2006.01)

(21) **International Application Number**: PCT/US2016/037422

(22) **International Filing Date**: 14 June 2016 (14.06.2016)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
62/175,916 15 June 2015 (15.06.2015) US

(71) **Applicant**: **CEPHEID** [US/US]; 904 Caribbean Drive, Sunnyvale, California 94089 (US).

(72) **Inventors**: **LAI, Edwin W.**; 904 Caribbean Drive, Sunnyvale, California 94089 (US). **KOHLWAY, Andrew**; 904 Caribbean Drive, Sunnyvale, California 94089 (US). **VANATTA, Reuel**; 904 Caribbean Drive, Sunnyvale, California 94089 (US). **HIGAUCHI, Russell**; 904 Caribbean Drive, Sunnyvale, California 94089 (US). **GALL, Alexander**; 904 Caribbean Drive, Sunnyvale, California 94089 (US). **KOCMOND, Kriszten**; 904 Caribbean Drive, Sunnyvale, California 94089 (US).

(74) **Agents**: **HUNTER, Tom** et al.; Weaver Austin Villeneuve & Sampson LLP, P.O. Box 70250, Oakland CA 94612-0250 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

(54) **Title**: INTEGRATED PURIFICATION AND MEASUREMENT OF DNA METHYLATION AND CO-MEASUREMENT OF MUTATIONS AND/OR MRNA EXPRESSION LEVELS IN AN AUTOMATED REACTION CARTRIDGE

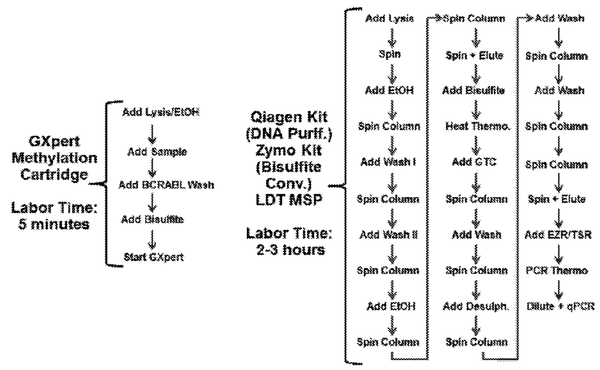


Fig. 23A

(57) **Abstract**: In various embodiments methods of determining methylation of DNA are provided. In one illustrative, but non-limiting embodiment the method comprises i) contacting a biological sample comprising a nucleic acid to a first matrix material comprising a first column or filter where said matrix material binds and/or filters nucleic acids in said sample and thereby purifies the DNA; ii) eluting the bound DNA from the first matrix material and denaturing the DNA to produce eluted denatured DNA; iii) heating the eluted DNA in the presence of bisulfite ions to produce a deaminated nucleic acid; iv) contacting said deaminated nucleic acid to a second matrix material comprising a second column to bind said deaminated nucleic acid to said second matrix material; v) desulfonating the bound deaminated nucleic acid and/or simultaneously eluting and desulfonating the nucleic acid by contacting the deaminated nucleic acid with an alkaline solution to produce a bisulfite converted nucleic acid; vi) eluting said bisulfite converted nucleic acid from said second matrix material; and vii) performing methylation specific PCR and/or nucleic acid sequencing, and/or high resolution melting analysis (HRM) on said bisulfite-converted nucleic acid to determine the methylation of said nucleic acid, wherein at least steps iv) through vi) are performed in a single reaction cartridge.

WO 2016/205233 A3

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
2 February 2017

INTEGRATED PURIFICATION AND MEASUREMENT OF DNA METHYLATION AND CO-MEASUREMENT OF MUTATIONS AND/OR MRNA EXPRESSION LEVELS IN AN AUTOMATED REACTION CARTRIDGE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of USSN 62/175,916, filed on June 15, 2015, which is incorporated herein by reference in its entirety for all purposes.

STATEMENT OF GOVERNMENTAL SUPPORT

[Not Applicable]

BACKGROUND

[0002] The genomes of higher eukaryotes contain the modified nucleoside 5-methyl cytosine (5-meC). This modification is usually found as part of the dinucleotide CpG in which cytosine is converted to 5-methylcytosine in a reaction that involves flipping a target cytosine out of an intact double helix and transfer of a methyl group from S-adenosylmethionine by a methyltransferase enzyme (see, *e.g.*, Klimasauskas *et al.* (1994) Cell 76: 357-369). This enzymatic conversion is the primary epigenetic modification of DNA known to exist in vertebrates and is essential for normal embryonic development (*see, e.g.,* Bird (1992) *Cell* 70: 5-8; Laird and Jaenisch (1994) *Human Mol. Genet.* 3: 1487-1495; and Li *et al.* (1992) *Cell* 69: 915-926).

[0003] In eukaryotes, DNA methylation regulates normal cellular processes such as genomic imprinting, chromosomal instability, and X-chromosome inactivation. Typically, DNA methylation occurs at the fifth carbon position of cytosine at dinucleotide 5'-CpG-3' sites in or near gene promoters termed CpG islands or shores. Methylation controls gene expression by down-regulating transcription either by directly inhibiting transcriptional machinery or indirectly through the recruitment of chromatin remodeling proteins. Chromosomal methylation patterns change dynamically during embryonic development, and the correct methylation patterns have to be maintained throughout an individual's lifetime. Changes in methylation patterns are linked to aging, and errors in DNA methylation are among the earliest changes that occur during oncogenesis. Thus, the detection of methylation at gene promoters is important, *inter alia*, for diagnosing and/or monitoring patients with cancer.

[0004] Epigenetic alterations, including DNA methylation, interrupt the DNA-RNA-protein axis which describes how genetic information is transcribed into messenger RNAs (mRNAs). The correlation between genomic DNA variation, mRNA copy numbers and protein levels may be described by DNA methylation levels. Thus co-measurement of DNA methylation levels and corresponding down-stream mRNA levels can be important to understanding the mechanism of epigenetic cellular regulation.

[0005] Several methods have been developed to detect and quantify methylation efficiently and accurately. The most common technique is the bisulfite conversion method which converts unmethylated cytosines to uracil. Once converted, the methylation profile of DNA can be determined by standard PCR techniques, sequencing methods, and the like.

[0006] There are several DNA Methylation kits suitable for bisulfite conversion and DNA cleanup (*e.g.*, EZ DNA Methylation™ kits from Zymo Research). Most kits involve several steps, reagents, and incubation times and often require purified DNA before conversion although some kits can utilize tissue or plasma/serum as starting material.

[0007] Typically the bisulfite conversion process requires at least four steps: 1) DNA Denaturation; 2) Bisulfite Incubation; 3) DNA Purification; and 4) Desulphonation. The final desulphonation step can be completed on-column or in solution followed by an ethanol precipitation. There are currently no methylation kits that allow a user to complete the entire process- DNA purification, bisulfite incubation, desulphonation, second DNA purification, and methylation-specific PCR all in one step.

SUMMARY

[0008] Various embodiments contemplated herein may comprise, but need not be limited to, one or more of the following:

[0009] Various embodiments contemplated herein may include, but need not be limited to, one or more of the following:

[0010] Embodiment 1: A method of determining the methylation state of a nucleic acid, said method comprising:

[0011] i) contacting a biological sample comprising a nucleic acid to a first matrix material comprising a first column or filter where said matrix material binds and/or filters nucleic acids in said sample and thereby purifies the DNA;

[0012] ii) eluting the bound DNA from the first matrix material and denaturing the DNA to produce eluted denatured DNA;

[0013] iii) heating the eluted DNA in the presence of a bisulfite reagent to produce a deaminated nucleic acid;

[0014] iv) contacting said deaminated nucleic acid to a second matrix material comprising a second column to bind said deaminated nucleic acid to said second matrix material;

[0015] v) desulfonating the bound deaminated nucleic acid and/or simultaneously eluting and desulfonating the nucleic acid by contacting the deaminated nucleic acid with an alkaline solution to produce a converted (*e.g.*, bisulfite converted) nucleic acid;

[0016] vi) eluting said bisulfite converted nucleic acid from said second matrix material; and

[0017] vii) performing methylation specific PCR and/or nucleic acid sequencing, and/or high resolution melting analysis (HRM) on said converted nucleic acid to determine the methylation of said nucleic acid, wherein at least steps iv) through vi) are performed in a single reaction cartridge.

[0018] Embodiment 2: The method of embodiment 1, wherein at least steps iv) through vi) are performed in a single reaction cartridge.

[0019] Embodiment 3: The method of embodiment 1, wherein at least steps iii) through vi) are performed in a single reaction cartridge.

[0020] Embodiment 4: The method of embodiment 1, wherein at least steps ii) through vi) are performed in a single reaction cartridge.

[0021] Embodiment 5: The method of embodiment 1, wherein at least steps i) through vi) are performed in a single reaction cartridge.

[0022] Embodiment 6: The method according to any one of embodiments 1-5, wherein step vii is performed in the same reaction cartridge.

[0023] Embodiment 7: The method according to any one of embodiments 1-6, wherein said first matrix material and said second matrix material are the same material forming the same column.

[0024] Embodiment 8: The method according to any one of embodiments 1-7, wherein said first matrix material and said second matrix material form different columns.

[0025] Embodiment 9: The method according to any one of embodiments of embodiment 1-8, wherein said methylation specific PCR, when performed, is performed in said cartridge.

[0026] Embodiment 10: The method according to any one of embodiments 1-9, wherein said nucleic acid sequencing, when performed, is performed in said cartridge or in a device coupled to said cartridge.

[0027] Embodiment 11: The method according to any one of embodiments 1-10, wherein said cartridge comprises a column comprising said first matrix material, a sample receiving chamber, a temperature controlled channel or chamber, a plurality of chambers containing reagents and/or buffers, and when in use at least one of said chambers contains a desulfonation/elution buffer, and wherein said cartridge optionally comprises a second column comprising said second matrix material.

[0028] Embodiment 12: The method of embodiment 11, wherein, when in use, at least one of said chambers contains a reagent that provides bisulfite ions.

[0029] Embodiment 13: The method according to any one of embodiments 11-12, wherein said second column is absent.

[0030] Embodiment 14: The method according to any one of embodiments 11-13, wherein said second column is present.

[0031] Embodiment 15: The method according to any one of embodiments 11-14, wherein said cartridge comprises a thermocycling channel or chamber in addition to said temperature controlled channel or chamber.

[0032] Embodiment 16: The method according to any one of embodiments 11-14, wherein said temperature controlled channel or chamber is a thermocycling channel or chamber.

[0033] Embodiment 17: The method according to any one of embodiments 11-16, wherein said cartridge comprises one or more chambers containing one or more reagents selected from the group consisting of methylation specific PCR primers, methylation specific PCR probes, PCR enzyme(s), and PCR reaction buffer.

[0034] Embodiment 18: The method of embodiment 17, wherein said cartridge comprises one or more chambers containing one or more primers and probes for detection of methylation of a forward strand of a bisulfite-converted DNA.

[0035] Embodiment 19: The method according to any one of embodiments 17-18, wherein said cartridge comprises one or more chambers containing one or more primers and probes for detection of methylation of a reverse strand of a bisulfite-converted DNA.

[0036] Embodiment 20: The method according to any one of embodiments 11-19, wherein said sample receiving chamber, said column(s), said plurality of chambers, and when present, said temperature controlled channel or chamber and/or thermocycling channel or chamber, are selectively in fluid communication.

[0037] Embodiment 21: The method of embodiment 20, wherein said sample receiving chamber, said column(s), said plurality of chambers, and when present, said thermocycling channel or chamber, are selectively in fluid communication by microfluidic channels and valves.

[0038] Embodiment 22: The method of embodiment 20, wherein said sample receiving chamber, said column(s), said plurality of chambers, and when present, said thermocycling channel or chamber or a port into said thermocycling channel or chamber, are disposed around a central valve and selectively in fluid communication with a channel in said central valve, wherein said central valve is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with said central valve.

[0039] Embodiment 23: The method according to any one of embodiments 11-22, wherein said cartridge, when in use, comprises:

[0040] a first chamber containing a sample;

[0041] a second chamber containing a guanidinium thiocyanate-ethanol (GTC-EtOH) solution;

[0042] a third chamber containing a bisulfite reagent;

[0043] a fourth chamber containing a buffer;

[0044] a fifth chamber containing a rinse solution; and

[0045] a sixth chamber containing an elution/desulfonation reagent.

[0046] Embodiment 24: The method of embodiment 23, wherien first chamber contains said sample in a GTC-EtOH-Tween extraction/precipitation reagent.

[0047] Embodiment 25: The method according to any one of embodiments 23-24, wherein the GTC-ETOH-Tween buffer is added at or near the time the sample is placed into the cartridge.

[0048] Embodiment 26: The method according to any one of embodiments 23-25, wherein the bisulfite reagent is added to the cartridge at or near the time the sample is placed in the cartridge.

[0049] Embodiment 27: The method of embodiment 23, wherein the GTC-ETOH-Tween buffer is provided as a component of the cartridge.

[0050] Embodiment 28: The method according to any one of embodiments 23-25, wherein the bisulfite reagent is provided as a component of the cartridge.

[0051] Embodiment 29: The method according to any one of embodiments 11-28, wherein said cartridge comprises a seventh chamber containing PCR primers and/or probes and/or PCR enzymes.

[0052] Embodiment 30: The method according to any one of embodiments 11-29, wherein said cartridge comprises an eighth chamber also containing PCR primers and/or probes and/or PCR enzymes.

[0053] Embodiment 31: The method of embodiments 29-30, wherein said PCR primers, and/or probes, and/or enzymes are provided as beads.

[0054] Embodiment 32: The method according to any one of embodiments 1-31, wherein said biological sample comprises one or more samples selected from the group consisting of a cell, a tissue, and a biological fluid containing a nucleic acid.

[0055] Embodiment 33: The method of embodiment 32, wherein said biological sample comprises a biological fluid selected from the group consisting of whole blood, plasma, serum, saliva, mucus, urine, sputum, pancreatic juice, and cerebrospinal fluid.

[0056] Embodiment 34: The method of embodiment 32, wherein said biological sample comprises a sample selected from the group consisting of a tissue sample, a formalin fixed paraffin embedded (FFPE) tissue, fresh frozen tissue, fine needle aspirates (FNA), and a core biopsy.

[0057] Embodiment 35: The method according to any one of embodiments 1-34, wherein said method comprises contacting said biological sample with a lysis solution.

[0058] Embodiment 36: The method of embodiment 35, wherein said method comprises providing said sample in said sample receiving chamber and contacting said sample with an extraction/precipitation solution.

[0059] Embodiment 37: The method according to any one of embodiments 1-36, wherein said matrix material comprises a column material selected from the group consisting of glass or silica, an ion exchange resin, cellulose, and hydroxyapatite.

[0060] Embodiment 38: The method of embodiment 37, wherein said matrix material comprises glass.

[0061] Embodiment 39: The method according to any one of embodiments 1-38, wherein said bisulfite ion is provided as compound selected from the group consisting of ammonium bisulfite, sodium metabisulfite, potassium bisulfite, cesium bisulfite, and DABSO.

[0062] Embodiment 40: The method of embodiment 39, wherein said bisulfite ion is provided by ammonium bisulfite.

[0063] Embodiment 41: The method according to any one of embodiments 1-40, wherein said bisulfite is provided in a reagent mix comprising scavengers to prevent sulfite oxidation and/or catalysts.

[0064] Embodiment 42: The method of embodiment 41, wherein said bisulfite is provided in a reagent mix comprising scavengers selected from the group consisting of Trolox and hydroquinone.

[0065] Embodiment 43: The method according to any one of embodiments 41-42, wherein said bisulfite is provided in a reagent mix comprising polyamines as catalysts.

[0066] Embodiment 44: The method according to any one of embodiments 1-43, wherein said eluting the bound DNA comprises eluting and denaturing said DNA using a low concentration of potassium hydroxide or other base.

[0067] Embodiment 45: The method of embodiment 44, wherein said eluting the bound DNA comprises eluting and denaturing said DNA with an alkaline solution with a pH greater than about pH 10.5.

[0068] Embodiment 46: The method of embodiment 44, wherein said eluting the bound DNA comprises eluting and denaturing said DNA with an alkaline solution with a pH greater than about pH 12.

[0069] Embodiment 47: The method of embodiments 45-46, wherein said alkaline solution is a 10-15 mM KOH solution.

[0070] Embodiment 48: The method according to any one of embodiments 1-47, wherein said incubating the eluted DNA with bisulfite ions to produce a deaminated nucleic acid comprises incubating the DNA in an ammonium bisulfite solution having a concentration that ranges from about 6M to about 7M.

[0071] Embodiment 49: The method of embodiment 48, wherein said incubating the eluted DNA with bisulfite ions to produce a deaminated nucleic acid comprises incubating the DNA in an ammonium bisulfite solution having a concentration of about 6.5M.

[0072] Embodiment 50: The method of embodiment 49, wherein said incubating comprises transferring the DNA in a concentrated bisulfite solution into a temperature controlled channel or chamber in said cartridge and heating said mixture.

[0073] Embodiment 51: The method of embodiment 50, wherein said incubating comprises thermally cycling the concentrated bisulfite solution from a temperature of about 60°C to about 95°C.

[0074] Embodiment 52: The method according to any one of embodiments 1-51, wherein said contacting said deaminated nucleic acid to a second matrix material comprises mixing the DNA-bisulfite solution with fresh GTC-EtOH and dispensing the solution over said second matrix material.

[0075] Embodiment 53: The method of embodiment 52, wherein said method comprises washing the DNA in said second matrix material with fresh GTC-EtOH, and then a rinse solution.

[0076] Embodiment 54: The method of embodiment 53, wherein said rinse solution comprises PEG200.

[0077] Embodiment 55: The method according to any one of embodiments 1-54, wherein said desulfonating the bound deaminated nucleic acid comprises eluting the DNA from said second column with a high pH desulphonation buffer and incubating said solution.

[0078] Embodiment 56: The method of embodiment 55, wherein said incubating is for a period of time ranging from about 1 minute to about 1 hour, or from about 5 minutes to about 30 minutes, or from about 10 minutes to about 20 minutes, or for about 15 minutes.

[0079] Embodiment 57: The method of embodiments 55-56, wherein said high pH desulphonation/elution buffer comprises KOH.

[0080] Embodiment 58: The method according to any one of embodiments 55-57, wherein said incubation is in a chamber that previously held said high pH desulphonation buffer (*e.g.*, chamber 10).

[0081] Embodiment 59: The method according to any one of embodiments 1-58, wherein after the incubation with bisulfite ions, a temperature controlled channel or chamber is washed with a buffer to remove the residual bisulfite and neutralize pH.

[0082] Embodiment 60: The method according to any one of embodiments 1-59, wherein high resolution melting analysis (HRM) on said bisulfite-converted nucleic acid is performed to determine the methylation of said nucleic acid.

[0083] Embodiment 61: The method according to any one of embodiments 1-60, wherein nucleic acid sequencing of said bisulfite-converted nucleic acid is performed to determine the methylation of said nucleic acid.

[0084] Embodiment 62: The method according to any one of embodiments 1-60, wherein methylation specific PCR is performed to determine methylation of target nucleic acid sequences.

[0085] Embodiment 63: The method of embodiment 62, wherein said methylation specific PCR (MSP) is performed using primers specific for methylated sequences and/or primers specific for unmethylated sequences.

[0086] Embodiment 64: The method of embodiment 62, wherein said methylation specific PCR comprises a MethyLight protocol.

[0087] Embodiment 65: The method of embodiment 62, wherein TaqMan PCR reactions are performed with primers specific for bisulfite-converted methylated and/or unmethylated sequences.

[0088] Embodiment 66: The method according to any one of embodiments 62-65, wherein said MSP utilizes one or more fluorescent probes that are markers for amplified methylated sequences and/or one or more fluorescent probes that are markers for amplified unmethylated sequences.

[0089] Embodiment 67: The method of embodiment 66, wherein said fluorescent probes comprise a fluorescent reporter dye and a quencher dye where the probe provides a signal upon cleavage by 5' to 3' nuclease activity of Taq DNA polymerase.

[0090] Embodiment 68: The method according to any one of embodiments 66-67, wherein a methylation signal is determined by the combined signal for a plurality of probes each specific to a different methylated region in an amplified region of interest.

[0091] Embodiment 69: The method according to any one of embodiments 66-67, wherein a methylation signal is determined by a plurality of probes specific for the same methylated region in an amplified region of interest.

[0092] Embodiment 70: The method according to any one of embodiments 66-67, wherein said plurality of probes comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, or more probes.

[0093] Embodiment 71: The method according to any one of embodiments 66-67, wherein a methylation signal is determined by a single probe in the amplified region of interest.

[0094] Embodiment 72: The method according to any one of embodiments 66-71, wherein said probes are run in simplex or multiplex.

[0095] Embodiment 73: The method according to any one of embodiments 66-71, wherein said probes are run in a multiplex format.

[0096] Embodiment 74: The method according to any one of embodiments 66-73, wherein said probes are run as a nested PCR reaction.

[0097] Embodiment 75: The method according to any one of embodiments 66-74, wherein said PCR reaction comprises a bisulfite contamination control probe that that undergoes bisulfite-mediated cleavage during PCR if bisulfite is present in the reaction.

[0098] Embodiment 76: The method according to any one of embodiments 1-75, wherien PCR is performed for one or more mutated genes.

[0099] Embodiment 77: The method according to any one of embodiments 1-76, wherein PCR is performed for unconverted DNA as a control.

[0100] Embodiment 78: The method according to any one of embodiments 1-77, wherein PCR is performed for converted DNA as a control.

[0101] Embodiment 79: The method of embodiment 77, wherein PCR is performed for unconverted DNA where the unconverted DNA is a target for said method.

[0102] Embodiment 80: The method according to any one of embodiments 1-79, wherein a bisulfite reaction and a PCR reaction, or a desulfonation reaction and a PCR reaction, or a bisulfite reaction, a desulfonation reaction and a PCR reaction are all performed in the same reaction tube or chamber.

[0103] Embodiment 81: The method according to any one of embodiments 1-80, wherein said contacting a biological sample comprising a nucleic acid to a first matrix material comprises contacting a sample containing RNA to said first matrix material, where said matrix material binds said RNA thereby purifies the RNA.

[0104] Embodiment 82: The method of embodiment 81, wherein said method comprises eluting said RNA from said matrix material substantially independently of the DNA.

[0105] Embodiment 83: The method of embodiment 82, wherein the RNA is eluted from said first matrix material using a Tris buffered elution.

[0106] Embodiment 84: The method according to any one of embodiments 81-83, wherein said RNA is eluted and stored in a chamber.

[0107] Embodiment 85: The method according to any one of embodiments 81-84, wherein reverse transcription (RT) is performed on said RNA and qRT-PCR is performed to determine the level of target RNA sequences.

[0108] Embodiment 86: The method according to any one of embodiments 82-85, where the RNA fraction is used to elute the bisulfite converted nucleic acid from said second matrix material and mix with the bisulfite-converted DNA, or is mixed with eluted bisulfite-converted DNA.

[0109] Embodiment 87: The method of embodiment 86, wherein RT is performed on said RNA prior to, or after, combination with the bisulfite-converted DNA.

[0110] Embodiment 88: The method according to any one of embodiments 86-87, wherein qRT-PCR is performed for RT RNA in the mixture to determine the level of target RNA sequences and methylation specific PCR is performed on the mixture to determine methylation of target DNA sequences.

[0111] Embodiment 89: The method according to any one of embodiments 1-88, where methylation is determined for a promoter region of a gene selected from the group consisting of *MGMT. RASSF1A, ADAMTS1, BNC1, HIST1H3C, HOXB4, RASGRF2, TM6SF1*, and *AKR1B1*.

[0112] Embodiment 90: The method according to any one of embodiments 81-89, wherein the expression level of RNA is determined for a methyltransferase.

[0113] Embodiment 91: The method of embodiment 90, wherein the expression level of RNA is determined for a methyltransferase selected from the group consisting of DNMT1, DNMT2, DNMT3A, DNMT3B, and TNMT3L.

[0114] Embodiment 92: A cartridge for determining the methylation state of a nucleic acid, said cartridge comprising: a column comprising a first matrix material, a sample receiving chamber, a temperature controlled channel or chamber, a plurality of chambers containing reagents and/or buffers, and when in use at least one of said chambers contains a bisulfite reagent, and at least one of said chambers contains a desulphonation/elution buffer, and wherein said cartridge optionally comprises a second column comprising said second matrix material.

[0115] Embodiment 93: The cartridge of embodiment 92, wherein said cartridge, when in use, comprises a chamber containing a reagent comprising guanidinium thiocyanate ethanol (GTC-EtOH).

[0116] Embodiment 94: The cartridge according to any one of embodiments 92-93, wherein said second column is absent.

[0117] Embodiment 95: The cartridge according to any one of embodiments 92-93, wherein said second column is present.

[0118] Embodiment 96: The cartridge according to any one of embodiments 92-95, wherein said temperature controlled channel or chamber is a thermocycling channel or chamber.

[0119] Embodiment 97: The cartridge according to any one of embodiments 92-96, wherein said cartridge further comprises a second heating channel or chamber.

[0120] Embodiment 98: The cartridge according to any one of embodiment 92-97, wherein said bisulfite reagent comprises a compound selected from the group consisting of ammonium bisulfite, sodium metabisulfite, potassium bisulfite, cesium bisulfite, and DABSO.

[0121] Embodiment 99: The cartridge of embodiment 98, wherein said bisulfite reagent comprises ammonium bisulfite.

[0122] Embodiment 100: The cartridge according to any one of embodiments 92-99, wherein said bisulfite is provided in a reagent mix comprising scavengers to prevent sulfite oxidation and/or catalysts.

[0123] Embodiment 101: The cartridge of embodiment 100, wherein said bisulfite is provided in a reagent mix comprising scavengers selected from the group consisting of Trolox and hydroquinone.

[0124] Embodiment 102: The cartridge according to any one of embodiments 100-101, wherein said bisulfite is provided in a reagent mix comprising polyamines as catalysts.

[0125] Embodiment 103: The cartridge according to any one of embodiments 92-102, wherein said first matrix material and/or said second matrix material, when present, comprises a material is selected from the group consisting of glass or silica, an ion exchange resin, and hydroxyapatite.

[0126] Embodiment 104: The cartridge according to any one of embodiments 92-103, wherein said cartridge comprises one or more chambers containing one or more reagents selected from the group consisting of methylation specific PCR primers, methylation specific PCR probes, PCR enzyme(s), and PCR reaction buffer.

[0127] Embodiment 105: The cartridge of embodiment 104, wherein said cartridge contains at least two chambers containing one or more reagents selected from the group consisting of methylation specific PCR primers, methylation specific PCR probes, PCR enzyme(s), and PCR reaction buffer.

[0128] Embodiment 106: The cartridge according to any one of embodiments 92-105, wherein said cartridge contains at least one chamber containing primers and probes for detection of methylation of a forward strand of a converted DNA.

[0129] Embodiment 107: The cartridge according to any one of embodiments 92-106, wherein said cartridge contains at least one chamber containing primers and probes for detection of methylation of a reverse strand of a converted DNA.

[0130] Embodiment 108: The cartridge according to any of embodiments 104-107, wherein said PCR primers, and/or probes, and/or enzymes are provided as beads.

[0131] Embodiment 109: The cartridge according to any one of embodiments 92-108, wherein said sample receiving chamber, said column(s), said plurality of chambers, and said temperature-controlled heating channel or chamber, are selectively in fluid communication.

[0132] Embodiment 110: The cartridge of embodiment 109, wherein said sample receiving chamber, said column(s), said plurality of chambers, and said temperature controlled channel or chamber, are selectively in fluid communication by microfluidic channels and valves.

[0133] Embodiment 111: The cartridge of embodiment 109, wherein said sample receiving chamber, said column(s), said plurality of chambers, and said temperature controlled channel or chamber or a port into said temperature controlled channel or chamber, are disposed around a central valve and selectively in fluid communication with a channel in said central valve, wherein said central valve is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with said central valve.

[0134] Embodiment 112: The cartridge according to any one of embodiments 92-111, wherein said cartridge is configured so that, when in use, said cartridge comprises:

[0135] a first chamber containing a sample;

[0136] a second chamber containing a guanidinium thiosulfate-ethanol (GTC-EtOH) solution;

[0137] a third chamber containing a bisulfite reagent;

[0138] a fourth chamber containing a buffer;

[0139] a fifth chamber containing a rinse solution; and

[0140] a sixth chamber containing an elution/desulphonation reagent.

[0141] Embodiment 113: The cartridge of embodiment 112, wherein said first chamber contains said sample in a GTC-EtOH-Tween extraction/precipitation reagent.

[0142] Embodiment 114: The cartridge according to any one of embodiments 92-113, wherein the cartridge is configured for the bisulfite reagent to be added to the cartridge at or near the time the sample is placed in the cartridge.

[0143] Embodiment 115: The cartridge according to any one of embodiments 92-113, wherein the bisulfite reagent is provided as a component of the cartridge.

[0144] Embodiment 116: The cartridge according to any one of embodiments 92-115, wherein the cartridge is configured for addition of GTC-ETOH-Tween buffer at or near the time the sample is placed into the cartridge.

[0145] Embodiment 117: The cartridge according to any one of embodiments 92-115, wherein the GTC-ETOH-Tween buffer is provided as a component of the cartridge.

[0146] Embodiment 118: The cartridge according to any one of embodiments 92-117, wherein said cartridge comprises a seventh chamber containing PCR primers and/or probes and/or PCR enzymes.

[0147] Embodiment 119: The cartridge according to any one of embodiments 92-118, wherein said cartridge comprises an eighth chamber also containing PCR primers and/or probes and/or PCR enzymes.

[0148] Embodiment 120: The cartridge according to any one of embodiments 92-119, wherein said cartridge comprises one or more chambers containing primers specific for bisulfite-converted methylated and/or unmethylated sequences.

[0149] Embodiment 121: The cartridge according to any one of embodiments 92-120, wherein said cartridge comprises one or more chambers containing reagents for TaqMan PCR reactions.

[0150] Embodiment 122: The cartridge according to any one of embodiments 92-121, wherein said cartridge comprises one or more chambers containing one or more fluorescent probes that are markers for amplified methylated sequences and/or one or more fluorescent probes that are markers for amplified unmethylated sequences.

[0151] Embodiment 123: The cartridge of embodiment 122, wherein said probes comprise a fluorescent reporter dye and a quencher dye, where the probes provides a signal upon cleavage by the 5' to 3' nuclease activity of Taq DNA polymerase.

[0152] Embodiment 124: The cartridge according to any one of embodiments 122-123, wherein said cartridge comprises a plurality of probes each specific to a different methylated region in an amplified region of interest.

[0153] Embodiment 125: The cartridge according to any one of embodiments 122-123, wherein said cartridge comprises a single probe specific to a methylated region in an amplified region of interest.

[0154] Embodiment 126: The cartridge according to any one of embodiments 122-123, wherein said cartridge comprises a plurality of probes each specific to the same methylated region in an amplified region of interest.

[0155] Embodiment 127: The cartridge according to any one of embodiments 92-126, wherein said cartridge contains primers and/or probes to determine methylation of a promoter region of a gene selected from the group consisting of *MGMT, RASSF1A, ADAMTS1, BNC1, HIST1H3C, HOXB4, RASGRF2, TM6SF1*, and *AKR1B1*.

[0156] Embodiment 128: The cartridge according to any one of embodiments 92-126, wherein said cartridge contains one or more primers shown in Tables 5, 9, or 10, and/or one or more probes shown in Tables 5, 9, or 10.

[0157] Embodiment 129: The cartridge of embodiment 128, wherein said cartridge contains the following probes and primers for determining methylation of *MGMT* using a nested PCR reaction:

[0158] an external forward primer (248b) comprising the nucleotide sequence GTTTT(T*)AGAAYG(T*)TTTGYGTTT (SEQ ID NO:263);

[0159] an external reverse primer (249b) comprising the nucleotide sequence: AAAAAAC(T*)CCRCACTCTTCC (SEQ ID NO:265);

[0160] an internal forward primer (250) comprising the nucleotide sequence TTTCGACGTTCGTAGGTTTTCGC (SEQ ID NO:266);

[0161] an internal reverse primer (251) comprising the nucleotide sequence GCACTCTTCCGAAAACGAAACG (SEQ ID NO:267); and

[0162] a probe (252a) comprising the nucleotide sequence fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-blocker (SEQ ID NO: 268).

[0163] Embodiment 130: The cartridge according to any one of embodiments 128-129, wherein said cartridge contains the following probes and primers for determining methylation of ACTB (*e.g.*, as a control) using a nested PCR reaction:

[0164] an external forward primer (102) comprising the nucleotide sequence: GTGATGGAGGAGGTTTAGTAAGTT (SEQ ID NO:103);

[0165] an external reverse primer (103) comprising the nucleotide sequence: CCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO:104);

[0166] an internal forward primer (148) comprising the nucleotide sequence: GGTTTAGTAAGTTTTTTGGATTGTG (SEQ ID NO:149);

[0167] an internal reverse primer (149) comprising the nucleotide sequence: CCTTAAAAATTACAAAAACCACAAC (SEQ ID NO:150); and

[0168] a probe (178) comprising the nucleotide sequence: fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-blocker (SEQ ID NO:179).

[0169] Embodiment 131: The cartridge according to any one of embodiments 92-130, wherein the cartridge is configured for determination of the expression level of RNA for a methyltransferase.

[0170] Embodiment 132: The cartridge of embodiment 131, wherein said methyltransferases is selected from the group consisting of DNMT1, DNMT2, DNMT3A, DNMT3B, and TNMT3L.

[0171] Embodiment 133: A system for determining the methylation of a nucleic acid in a biological sample, said system comprising: an enclosure configured to contain one or more sample processing modules, each sample processing module configured to hold a removable cartridge according to any one of embodiments 92-132; where said system is configured to operate the sample processing modules to perform sample processing to determine methylation of one or more target nucleic acids and optionally to determine the level of one or more target DNA sequences within a corresponding removable sample cartridge, wherein said processing on a sample within the corresponding removable sample cartridge performs a method according to any one of embodiments 1-91.

[0172] Embodiment 134: The system of embodiment 133, wherein said system is configured to contain one sample processing module.

[0173] Embodiment 135: The system of embodiment 133, wherein said system is configured to contain at least two sample processing modules, or at least 4 sample processing modules, or at least 8 sample processing modules, or at least 12 sample processing modules, or at least 16 sample processing modules, or at least 20 sample processing modules, or at least 24 sample processing modules, or at least 28 sample processing modules, or at least 32 sample processing modules, or at least 64 sample processing modules, or at least 128 sample processing modules.

[0174] Embodiment 136: The system according to any one of embodiments 133-135, wherein said modules comprise one or more heating plates to heat a temperature controlled chamber or channel in said cartridge.

[0175] Embodiment 137: The system according to any one of embodiments 133-136, wherein said modules comprise a fan configured to cool a temperature controlled channel or chamber in said cartridge.

[0176] Embodiment 138: The system according to any one of embodiments 133-137, wherein said modules comprise circuitry to pass information (*e.g.*, optical information) to a computer for analysis.

[0177] Embodiment 139: The system according to any one of embodiments 133-138, wherein said modules comprise optical blocks to provide excitation and/or detection of one or more optical signals produced by reactions in said cartridge.

[0178] Embodiment 140: The system according to any one of embodiments 133-139, wherein said system is configured to operate said cartridge to perform a method according to any one of embodiments 1-91.

[0179] Embodiment 141: The system according to any one of embodiments 133-139, wherein said system is configured to operate said cartridge to: bind a sample to a column; elute DNA from the column and combine said DNA with a conversion reagent; heat the DNA/conversion reagent solution in a reaction chamber or tube to produce converted DNA; bind the converted DNA to a column; desulphonate and elute the DNA from the column; and perform PCR on the eluted desulphonated DNA in a reaction chamber or tube.

[0180] Embodiment 142: The system of embodiment 141, wherein said PCR is performed in the same reaction chamber or tube where the DNA/conversion reagent solution was previously heated.

[0181] Embodiment 143: A cartridge for sample preparation, said cartridge comprising: a channel or chamber comprising an affinity matrix that binds DNA, a plurality of chambers disposed around a central valve assembly and selectively in fluid communication with said central valve assembly where said central valve assembly is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with said central valve wherein said plurality of chambers comprises: a chamber configured to receive up to about 5 ml or up to about 4 ml of sample solution; a chamber containing PEG; a chamber containing GTC-EtOH; a chamber containing an alkaline solution; and a chamber containing a buffer.

[0182] Embodiment 144: The cartridge of embodiment 143, wherein said plurality of chambers further comprises a chamber containing a bisulfite reagent.

[0183] Embodiment 145: The cartridge according to any one of embodiments 143-144, wherein said plurality of chambers comprises a chamber containing a GTC-ethanol wash solution.

[0184] Embodiment 146: The cartridge of embodiment 145, wherein said GTC-ethanol wash solution comprises 1.25M guanidinium thiocyanate, 25 mM Tris pH 7.0, and 50% ethanol.

[0185] Embodiment 147: The cartridge according to any one of embodiments 143-146, wherein said PEG comprises PEG200.

[0186] Embodiment 148: The cartridge according to any one of embodiments 143-147, wherein said alkaline solution comprises KOH.

[0187] Embodiment 149: The cartridge according to any one of embodiments 143-148, wherein said buffer comprises Tris.

[0188] Embodiment 150: The cartridge according to any one of embodiments 143-149. wherein said plurality of chambers comprises a chamber containing beads comprising one or more PCR primers and/or probes.

[0189] Embodiment 151: The cartridge according to any one of embodiments 143-150, wherein said chamber containing PEG contains about 1 ml of PEG.

[0190] Embodiment 152. The cartridge according to any one of embodiments 143-151, wherein said chamber containing an alkaline solution contains about 500 µL of solution.

[0191] Embodiment 153: The cartridge according to any one of embodiments 143-152, wherein said chamber containing GTC-EtOH contains about 2 ml GTC-EtOH.

[0192] Embodiment 154: The cartridge according to any one of embodiments 143-153, wherein said chamber containing a buffer contains about 2 mL of buffer.

[0193] Embodiment 155: A high volume sample preparation (HVSP), said cartridge comprising: a channel or chamber comprising an affinity matrix that binds DNA, a plurality of chambers disposed around a central valve assembly and selectively in fluid communication with said central valve assembly where said central valve assembly is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with said central valve wherein said plurality of chambers comprises: at least two different chambers each configured to receive up to about 4.5 ml of sample solution; a chamber containing PEG; a chamber containing an alkaline solution; and a chamber containing a buffer.

[0194] Embodiment 156: The cartridge of embodiment 155, wherein said plurality of chambers comprises at least three different chambers each configured to receive up to 4 ml of sample solution.

[0195] Embodiment 157: The cartridge according to any one of embodiments 155-156, wherein said PEG comprises PEG200.

[0196] Embodiment 158: The cartridge according to any one of embodiments 155-157, wherein said basic solution comprises KOH.

[0197] Embodiment 159: The cartridge according to any one of embodiments 155-158, wherein said buffer comprises Tris.

[0198] Embodiment 160: The cartridge according to any one of embodiments 155-159, wherein said plurality of chambers comprises a chamber containing a wash solution.

[0199] Embodiment 161: The cartridge of embodiment 160, wherein said wash solution comprise 1.25M guanidinium thiocyanate, 25 mM Tris pH 7.0, and 50% ethanol.

[0200] Embodiment 162: The cartridge according to any one of embodiments 155-161, wherein said cartridge comprises a chamber configured for removal of a processed sample.

[0201] Embodiment 163: The cartridge according to any one of embodiments 155-162, wherein said sample chambers, when in use contain sample solution, GTC and isopropanol.

[0202] Embodiment 164: The cartridge of embodiment 163, wherein said sample chambers, when in use contain sample solution, GTC and isopropanol in substantially equal volumes.

[0203] Embodiment 165: The cartridge according to any one of embodiments 155-164 wherein said cartridge, when in use, comprises 4 ml of sample solution disposed in each of said chambers configured to receive a sample.

[0204] Embodiment 166: The cartridge according to any one of embodiments 155-165, wherein said cartridge provides DNA or RNA recovery that is substantially linear with respect to the sample volume between 0.5 ml and about 4 ml of sample.

[0205] Embodiment 167: The cartridge according to any one of embodiments 155-166, wherein said cartridge contains or is configured to receive a conversion reagent.

[0206] Embodiment 168: The cartridge of embodiment 167, wherein said cartridge, when in use, performs a bisulfite conversion of DNA.

[0207] Embodiment 169: A lysis solution for preparation of a DNA sample from serum or plasma, said lysis solution comprising: GTC, a buffer, a detergent, and optionally an anti-foaming agent.

[0208] Embodiment 170: The lysis solution of embodiment 169, wherein said lysis solution for serum or plasma comprises GTC, Tris pH 7.0, Tween 20, and antifoam SE15.

[0209] Embodiment 171: The lysis solution of embodiment 170, wherein said lysis solution for serum or plasma comprises about 4.5M GTC, about 45mM Tris pH 7.0, about 1% Tween20, and about 0.01% Antifoam SE15.

[0210] Embodiment 172: A lysis solution for preparation of a DNA sample from an FFPE sample.

[0211] Embodiment 173: The lysis solution of embodiment 172, wherein said lysis solution for FFPE samples comprises a buffer, a detergent, NaCl, $MgCl_2$, a chelating agent, antifoam SE15, and sodium azide.

[0212] Embodiment 174: The lysis solution of embodiment 173, wherein said lysis solution for FFPE samples comprises about 1% Tween20, about 400mM NaCl, about 25mM EDTA, about 10mM $MgCl_2$, about 50mM HEPES pH 7.2, about 0.01% antifoam SE15, and about 0.01% sodium azide.

[0213] Embodiment 175: A kit for the determination of DNA methylation, said kit comprising: a container containing a cartridge for determining the methylation state of a nucleic acid according to any one of embodiments 92-136.

[0214] Embodiment 176: The kit of embodiment 175, wherein said kit further comprises a container containing a lysis solution.

[0215] Embodiment 177: The kit of embodiment 176, wherein said lysis solution is a lysis solution for serum or plasma according to any one of embodiments 169-171.

[0216] Embodiment 178: The kit of embodimentx 176, wherein said lysis solution is a lysis solution for an FFPE sample according to any one of embodiments 172-174.

[0217] Embodiment 179: The kit according to any one of embodiments 175-178, wherein said kit comprises a container containing proteinase K.

[0218] Embodiment 180: The kit according to any one of embodiments 175-179, wherein said kit comprises a conversion reagent in said cartridge or in a container separate from the cartridge.

[0219] Embodiment 181: The kit of embodiment 180, wherein said kit comprises said conversion reagent in a container separate from the cartridge.

[0220] Embodiment 182: The kit of embodiment 180, wherein said kit comprises said conversion reagent is provided in a chamber of the cartridge.

[0221] Embodiment 183: The according to any one of embodiments 180-182, wherein said conversion reagent comprises a compound selected from the group consisting of sodium metabisulfite, potassium bisulfite, cesium bisulfite, ammonium bisulfite, and DABSO.

[0222] Embodiment 184: The kit of embodiment 183, wherein said conversion reagent comprises ammonium bisulfite.

[0223] Embodiment 185: The kit according to any one of embodiments 175-184, wherein said kit comprises a container containing a sample processing reagent.

[0224] Embodiment 186: The kit of embodiment 185, wherein said sample processing reagent comprises guanidium thiocyanate.

[0225] Embodiment 187: The kit according to any one of embodiments 185-186, wherein said sample processing reagent comprise ethanol.

[0226] Embodiment 188: The kit according to any one of embodiments 175-187, wherein said kit comprises a container containing a cartridge for sample preparation according to any one of embodiments 155-166.

[0227] Embodiment 189: The kit according to any one of embodiments 175-188, wherein said kit contains instructional materials teaching the use of said cartridge for the determination of DNA methylation.

[0228] Embodiment 190: A cartridge for the detection of methylation markers of a cancer, said cartridge comprising: a plurality of chambers and a thermocycling channel or chamber, wherein said plurality of chambers and a port into said thermocycling channel or chamber are disposed around a central valve assembly and selectively in fluid communication with said central valve assembly where said central valve assembly is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber or port in fluid communication with said central valve wherein said plurality of chambers comprises: a sample receiving chamber; a chamber containing or configured to receive a bisulfite reagent; a chamber containing a wash solution; a chamber containing a Tris buffer; a chamber containing an alkaline solution comprising KOH; a chamber containing beads that provide a PCR master mix; and a chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters whose methylation state is a marker for a cancer.

[0229] Embodiment 191: The cartridge of embodiment 190, wherein said plurality of chambers comprises a chamber disposed to receive waste solutions.

[0230] Embodiment 192: The cartridge according to any of embodiments 190-191, wherein said bisulfite reagent comprises a compound selected from the group consisting of sodium metabisulfite, potassium bisulfite, cesium bisulfite, ammonium bisulfite, and DABSO.

[0231] Embodiment 193: The cartridge of embodiment 192, wherein said bisulfite reagent comprises ammonium bisulfite.

[0232] Embodiment 194: The cartridge according to any of embodiments 190- 193, wherein said wash solution comprises 1.25M GTC, 25 mM Tris pH 7.0, and 50% ethanol.

[0233] Embodiment 195: The cartridge according to any of embodiments 190-194, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of one or more gene promoters whose methylation state is a marker for a cancer selected from the group consisting of breast cancer, pancreatic cancer, prostate cancer, brain cancer, and lung cancer.

[0234] Embodiment 196: The cartridge of embodiment 195, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes for a nested PCR reaction.

[0235] Embodiment 197: The cartridge of embodiment 196, wherein said nested PCR comprises a first PCR reaction specific for converted DNA and a second PCR reaction specific for methylated CpGs.

[0236] Embodiment 198: The cartridge according to any one of embodiments 190-197, wherein said chamber containing beads that provide PCR primers and probes chamber contains beads that provide PCR primers and probes to detect methylation of a forward strand of converted DNA.

[0237] Embodiment 199: The cartridge according to any one of embodiments 190-198, wherein said chamber containing beads that provide PCR primers and probes chamber contains beads that provide PCR primers and probes to detect methylation of a reverse strand of converted DNA.

[0238] Embodiment 200: The cartridge according to any of embodiments 190-199, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoters of one or more genes selected from the group consisting of *RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2, TM6SF1, BRCA1, BNC1, ADAMTS1, CDO1, SOX17, TAC1, HOXA7,* and *MGMT.*

[0239] Embodiment 201: The cartridge according to any of embodiments 190-200, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of one or more gene promoters whose methylation state is a marker for pancreatic cancer.

[0240] Embodiment 202: The cartridge of embodiment 201, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoters of *ADAMTS1*, and/or *BNC1.*

[0241] Embodiment 203: The cartridge of embodiment 202, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *ADAMTS1.*

[0242] Embodiment 204: The cartridge according to any one of embodiments 202-203, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *BNC1.*

[0243] Embodiment 205: The cartridge of embodiment 202, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide one or more PCR primers and/or probes for *ADAMTS1* and/or *BNC1* shown in Tables 5, or 10.

[0244] Embodiment 206: The cartridge according to any of embodiments 190-200, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of one or more gene promoters whose methylation state is a marker for breast cancer.

[0245] Embodiment 207: The cartridge of embodiment 206, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoters of one, two, three, four, five, or all genes selected from the group consisting of *BRCA1, RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2,* and *TM6SF1.*

[0246] Embodiment 208: The cartridge of embodiment 207, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *BRCA1.*

[0247] Embodiment 209: The cartridge according to any one of embodiments 207-208, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *RASSF1A.*

[0248] Embodiment 210: The cartridge according to any one of embodiments 207-209, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *AKR1B1.*

[0249] Embodiment 211: The cartridge according to any one of embodiments 207-210, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *HOXB4.*

[0250] Embodiment 212: The cartridge according to any one of embodiments 207-211, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *HIST1H3C.*

[0251] Embodiment 213: The cartridge according to any one of embodiments 207-212, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *RASGRF2*.

[0252] Embodiment 214: The cartridge according to any one of embodiments 207-213, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *TM6SF1*.

[0253] Embodiment 215: The cartridge according to any one of embodiments 207-214, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide one or more PCR primers and/or one or more PCR probes shown in Tables 5, or 9.

[0254] Embodiment 216: The cartridge of embodiment 206, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoters of *BRCA1*.

[0255] Embodiment 217: The cartridge according to any of embodiments 190-200, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of one or more gene promoters whose methylation state is a marker for lung cancer.

[0256] Embodiment 218: The cartridge of embodiment 217, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoters of one, two, three, or all genes selected from the group consisting of *CDO1*, *SOX17*, *TAC1*, and *HOXA7*.

[0257] Embodiment 219: The cartridge of embodiment 218, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *CDO1*.

[0258] Embodiment 220: The cartridge according to any one of embodiments 218-219, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *SOX17*.

[0259] Embodiment 221: The cartridge according to any one of embodiments 218-220, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *TAC1*.

[0260] Embodiment 222: The cartridge according to any one of embodiments 218-221, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *HOXA7*.

[0261] Embodiment 223: The cartridge according to any of embodiments 190-200, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of one or more gene promoters whose methylation state is a marker for brain cancer.

[0262] Embodiment 224: The cartridge of embodiment 223, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide PCR primers and probes to detect methylation of the promoter of *MGMT*.

[0263] Embodiment 225: The cartridge of embodiment 224, wherein said chamber containing beads that provide PCR primers and probes to detect methylation of one or more gene promoters comprises beads that provide one or more PCR primers and/or probes for MGMT shown in Tables 5, or 10.

[0264] Embodiment 226: The cartridge of embodiment 225, wherein said cartridge contains the following probes and primers for determining methylation of *MGMT* using a nested PCR reaction:

[0265] an external forward primer (248b) comprising the nucleotide sequence GTTTT(T*)AGAAYG(T*)TTTGYGTTT (SEQ ID NO:263);

[0266] an external reverse primer (249b) comprising the nucleotide sequence AAAAAAC(T*)CCRCACTCTTCC (SEQ ID NO:265);

[0267] an internal forward primer (250) comprising the nucleotide sequence TTTCGACGTTCGTAGGTTTTCGC (SEQ ID NO:266);

[0268] an internal reverse primer (251) comprising the nucleotide sequence

GCACTCTTCCGAAAACGAAACG (SEQ ID NO:267); and

[0269] a probe (252a) comprising the nucleotide sequence fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-blocker (SEQ ID NO: 268).

[0270] Embodiment 227: The cartridge according to any one of embodiments 225-226, wherein said cartridge contains the following probes and primers for determining methylation of ACTB (*e.g.*, as a control) using a nested PCR reaction:

[0271] an external forward pri

mer (102) comprising the nucleotide sequence GTGATGGAGGAGGTTTAGTAAGTT (SEQ ID NO:103);

[0272] an external reverse primer (103) comprising the nucleotide sequence CCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO:104);

[0273] an internal forward primer (148) comprising the nucleotide sequence GGTTTAGTAAGTTTTTGGATTGTG (SEQ ID NO:149);

[0274] an internal reverse primer (149) comprising the nucleotide sequence CCTTAAAAATTACAAAACCACAAC (SEQ ID NO:150); and

[0275] a probe (178) comprising the nucleotide sequence fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-blocker (SEQ ID NO:179).

[0276] Embodiment 228: A method of preparing a sample of cfDNA from serum or plasma, said method comprising:

[0277] combining a proteinase K treated sample of serum or plasma with a lysis solution according to any one of embodiments 169-171, and an alcohol to form a sample solution;

[0278] loading said sample solution into a sample receiving chamber in a cartridge according to any one of embodiments 143-154, or into a sample receiving chamber in a cartridge according to any one of embodiments 155-168; and

[0279] operating said cartridge to bind DNA in said sample to said affinity matrix and then to wash and release said DNA from said matrix.

[0280] Embodiment 229: The method of embodiment 228, wherein said combining a proteinase K treated sample of serum or plasma comprises combining said sample, lysis solution and alcohol in proportions corresponding to about 1.3 ml proteinase K treated serum or plasma, 2.2 mL lysis solution; and about 1.5 ml alcohol.

[0281] Embodiment 230: The method according to any one of embodiments 228-229, wherein said alcohol comprises isopropanol.

[0282] Embodiment 231: The method according to any one of embodiments 228-230, wherein said sample comprises serum.

[0283] Embodiment 232: The method according to any one of embodiments 228-231, wherein said sample comprises plasma.

[0284] Embodiment 233: The method according to any one of embodiments 228-232, wherein said sample comprises serum.

[0285] Embodiment 234: The method according to any one of embodiments 228-233, wherein operating said cartridge comprises introducing said cartridge into a sample processing module in a system according to any one of embodiments 133-139.

[0286] Embodiment 235: The method according to any one of embodiments 228-234, wherein said method further comprises operating said cartridge to convert said DNA for methylation detection.

[0287] Embodiment 236: The method according to any one of embodiments 228-235, wherein said method further comprises operating said cartridge to perform one or more PCR reactions using said DNA or converted DNA a template.

[0288] Embodiment 237: The method according to any one of embodiments 228-234, wherein said loading comprises loading said sample solution into one or more sample receiving chambers in a cartridge according to any one of embodiments 155-165.

[0289] Embodiment 238: The method of embodiment 237, wherein said method further comprises transferring the released DNA to a second cartridge for methylation detection and/or PCR.

[0290] Embodiment 239: The method of embodiment 238, wherein said second cartridge is a cartridge according to any one of embodiments 92-132.

[0291] Embodiment 240: The method according to any one of embodiments 238-239, wherein said method further comprises operating said second cartridge to convert said DNA for methylation detection.

[0292] Embodiment 241: The method according to any one of embodiments 238-240, wherein said method further comprises operating said second cartridge to perform one or more PCR reactions using said DNA or converted DNA as a template.

[0293] Embodiment 242: The method according to any one of embodiments 238-241, wherein said operating said second cartridge comprises introducing said second cartridge into a sample processing module in a system according to any one of embodiments 133-139.

[0294] Embodiment 243: A method of preparing a DNA from an FFPE sample, said method comprising:

[0295] combining a formalin-fixed paraffin embedded sample with a lysis solution according to any one of embodiments 172-174;

[0296] heating said lysis solution containing said sample; adding an alcohol to said sample to form a sample solution; loading said sample solution into a sample receiving chamber in a cartridge according to any one of embodiments 143-154, or into a sample receiving chamber in a cartridge according to any one of embodiments 155-168; and

[0297] operating said cartridge to bind DNA in said sample to said affinity matrix and then to wash and release said DNA from said matrix.

[0298] Embodiment 244: The method of embodiment 243, wherein said heating comprises adding proteinase K to said sample and heating said sample.

[0299] Embodiment 245: The method of embodiment 244, wherein said heating comprises adding about 50 µL proteinase K to about 1.2 mL of FFPE lysis solution containing a FFPE sample.

[0300] Embodiment 246: The method according to any one of embodiments 243-245, wherein said heating comprises heating said lysis solution to a temperature ranging from about 50°C to about 60°C.

[0301] Embodiment 247: The method of embodiment 246, wherein said heating comprises heating said lysis solution to a temperature of about 56°C.

[0302] Embodiment 248: The method according to any one of embodiments 243-247, wherein said heating is for a period of time ranging up to about 4 hours, or up to about 5 hours, or up to about 6 hours.

[0303] Embodiment 249: The method of embodiment 248, wherein said heating is for about 4 hours.

[0304] Embodiment 250: The method according to any one of embodiments 243-249, wherein said alcohol comprises ethanol.

[0305] Embodiment 251: The method according to any one of embodiments 243-250, wherein said method comprises adding alcohol to said lysis solution in a volume ratio of about 1:1 lysis solution:alcohol.

[0306] Embodiment 252: The method according to any one of embodiments 243-251, wherein operating said cartridge comprises introducing said cartridge into a sample processing module in a system according to any one of embodiments 133-139.

[0307] Embodiment 253: The method according to any one of embodiments 243-252, wherein said method further comprises operating said cartridge to convert said DNA for methylation detection.

[0308] Embodiment 254: The method according to any one of embodiments 243-253, wherein said method further comprises operating said cartridge to perform one or more PCR reactions using said DNA or converted DNA as a template.

[0309] Embodiment 255: The method according to any one of embodiments 243-251, wherein said loading comprise loading said sample solution into one or more sample receiving chambers in a cartridge according to any one of embodiments 155-165.

[0310] Embodiment 256: The method of embodiment 255, wherein said method further comprises transferring the released DNA to a second cartridge for methylation detection and/or PCR.

[0311] Embodiment 257: The method of embodiment 256, wherein said second cartridge is a cartridge according to any one of embodiments 92-132.

[0312] Embodiment 258: The method according to any one of embodiments 256-257, wherein said method further comprises operating said second cartridge to convert said DNA for methylation detection.

[0313] Embodiment 259: The method according to any one of embodiments 256-258, wherein said method further comprises operating said second cartridge to perform one or more PCR reactions using said DNA or converted DNA as a template.

[0314] Embodiment 260: The method according to any one of embodiments 256-259, wherein said operating said second cartridge comprises introducing said second cartridge into a sample processing module in a system according to any one of embodiments 133-139.

[0315] Embodiment 261: A method of detecting a cancer, and/or staging a cancer, and/or detecting the predisposition to a cancer in a subject, said method comprising:

[0316] providing a biological sample from said subject, wherein said biological sample comprises a DNA;

[0317] utilizing a cartridge according to any one of claims 190-225 to detect methylation of one or more gene promoters in said DNA whose methylation state is a marker for a cancer, where an increase in methylation of said one or more gene promoters is indicative of the presence of a cancer or a predisposition to a cancer or a stage of a cancer or precancer.

[0318] Embodiment 262: The method of embodiment 261, wherein said subject is a human.

[0319] Embodiment 263: The method according to any one of embodiments 261-262, wherein said cancer is a cancer selected from the group consisting of breast cancer, pancreatic cancer, prostate cancer, brain cancer, a lung cancer, a B cell lymphoma, a bronchus cancer, a colorectal cancer, a stomach cancer, an ovarian cancer, a urinary bladder cancer, a brain or central nervous system cancer, a peripheral nervous system cancer, an esophageal cancer, a cervical cancer, a melanoma, a uterine or endometrial cancer, a cancer of the oral cavity or pharynx, a liver cancer, a kidney cancer, a biliary tract cancer, a small bowel or appendix cancer, a salivary gland cancer, a thyroid gland cancer, a adrenal gland cancer, an osteosarcoma, a chondrosarcoma, a liposarcoma, a testes cancer, and a malignant fibrous histiocytoma.

[0320] Embodiment 264: The method according to any one of embodiments 261-262, wherein said cancer is a cancer selected from the group consisting of breast cancer, pancreatic cancer, prostate cancer, brain cancer, a lung cancer.

[0321] Embodiment 265: The method according to any one of embodiments 261-264, wherein said sample comprise a sample from serum or plasma.

[0322] Embodiment 266: The method according to any one of embodiments 261-264, wherein said sample comprise an FFPE sample.

[0323] Embodiment 267: The method according to any one of embodiments 261-266, wherein said one or more gene promoters comprise the promoters of one or more genes selected from the group consisting of *RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2, TM6SF1, BRCA1, BNC1, ADAMTS1, CDO1, SOX17, TAC1, HOXA7,* and *MGMT*.

[0324] Embodiment 268: The method according to any one of embodiments 261-266, wherein said cancer is pancreatic cancer and said one or more gene promoters comprise the promoters of one, two, three, or four genes selected from the group consisting of *ADAMTS1*, and *BNC1*.

[0325] Embodiment 269: The method of embodiment 268, wherein said one or more gene promoters comprise the promoter of *ADAMTS1*.

[0326] Embodiment 270: The method according to any one of embodiments 268-269, wherein said one or more gene promoters comprise the promoter of *BNC1*.

[0327] Embodiment 271: The method according to any one of embodiments 261-266, wherein said cancer is breast cancer and said one or more gene promoters comprise the promoters of one, two, three, four, five, or all genes selected from the group consisting of *BRCA1, RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2,* and *TM6SF1*.

[0328] Embodiment 272: The method of embodiment 271, wherein said one or more gene promoters comprise the promoter of *BRCA1*.

[0329] Embodiment 273: The method according to any one of embodiments 271-272, wherein said one or more gene promoters comprise the promoter of *RASSF1A*.

[0330] Embodiment 274: The method according to any one of embodiments 271-273, wherein said one or more gene promoters comprise the promoter of *AKR1B1*.

[0331] Embodiment 275: The method according to any one of embodiments 271-274, wherein said one or more gene promoters comprise the promoter of *HOXB4*.

[0332] Embodiment 276: The method according to any one of embodiments 271-275, wherein said one or more gene promoters comprise the promoter of *HIST1H3C*.

[0333] Embodiment 277: The method according to any one of embodiments 271-276, wherein said one or more gene promoters comprise the promoter of *RASGRF2*.

[0334] Embodiment 278: The method according to any one of embodiments 271-277, wherein said one or more gene promoters comprise the promoter of *TM6SF1*.

[0335] Embodiment 279: The method according to any one of embodiments 261-266, wherein said cancer is breast cancer and said one or more gene promoters comprise the promoter of *BRCA1*.

[0336] Embodiment 280: The method according to any one of embodiments 261-266, wherein said cancer is lung cancer and said one or more gene promoters comprise the promoters of one, two, three, for all genes selected from the group consisting of *CDO1, SOX17, TAC1*, and *HOXA7*.

[0337] Embodiment 281: The method of embodiment 280, wherein said one or more gene promoters comprise the promoter of *CDO1*.

[0338] Embodiment 282: The method according to any one of embodiments 280-281, wherein said one or more gene promoters comprise the promoter of *SOX17*.

[0339] Embodiment 283: The method according to any one of embodiments 280-282, wherein said one or more gene promoters comprise the promoter of *TAC1*.

[0340] Embodiment 284: The method according to any one of embodiments 280-283, wherein said one or more gene promoters comprise the promoter of *HOXA7*.

[0341] Embodiment 285: The method according to any one of embodiments 261-266, wherein said cancer is brain cancer and said one or more gene promoters comprise the promoter of *MGMT*.

[0342] Embodiment 286: A method of converting cytosine residues in a DNA to uracil, while leaving 5-methylcytosine residues substantially unaffected, said method comprising:

[0343] contacting a sample comprising DNA with DABSO to convert said DNA;

[0344] desulfonating the converted DNA, to produce a DNA in which cytosine residues are converted to uracil, but 5-methylcytosine residues substantially unaffected.

[0345] Embodiment 287: The method of embodiment 286, wherein said contacting comprises contacting said DNA with DABSO at a concentration ranging from about 2 M up to about 5 M.

[0346] Embodiment 288: The method of embodiment 286, wherein said contacting comprises contacting said DNA with DABSO at a concentration of about 2.5 M.

[0347] Embodiment 289: The method according to any one of embodiments 286-288, wherein said DABSO is dissolved in an alkaline aqueous solution.

[0348] Embodiment 290: The method of embodiment 289, wherein said DABSO is dissolved in a solution comprising KOH.

[0349] Embodiment 291: The method according to any one of embodiments 286-290, wherein said contacting comprises heating the DABSO/DNA solution to a temperature ranging from about 55°C to about 90°C.

[0350] Embodiment 292: The method according to any one of embodiments 286-291, wherein said DABSO is reacted with the DNA for a period of time ranging from about 15 minutes up to about 90 minutes.

[0351] Embodiment 293: The method according to any one of embodiments 286-292, wherein said desulfonating comprises contacting said converted DNA with an alkaline reagent.

[0352] Embodiment 294: The method of embodiment 293, wherein said alkaline reagent comprises KOH.

[0353] Embodiment 295: The method according to any one of embodiments 286-294, wherein said conversion and/or desulphonation is performed on the DNA bound to a column.

[0354] Embodiment 296: The method according to any one of embodiments 286-294, wherein said conversion and/or desulphonation is performed on the DNA in solution.

[0355] Embodiment 297: A method of analyzing DNA methylation, said method comprising:

[0356] providing a DNA sample;

[0357] converting DNA in said sample according to the method of any one of embodiments 286-296; and

[0358] performing methylation specific PCR and/or nucleic acid sequencing, and/or high resolution melting analysis (HRM) on the converted nucleic acid to determine the methylation of said nucleic acid.

[0359] Embodiment 298: The method of embodiment 297, wherein said providing a DNA sample comprises preparing a sample according to any one of embodiments 228-234 or according to any one of embodiments 243-252.

[0360] Embodiment 299: A kit for detection of methylation state of a DNA, said kit comprising:

[0361] a container containing a conversion reagent comprising DABSO; and

[0362] a container containing a desulphonation reagent.

[0363] Embodiment 300: The kit of embodiment 299, wherein said kit comprises a column comprising an affinity matrix.

[0364] Embodiment 301: The kit according to any one of embodiments 299-300, wherein said kit comprises a container containing a binding buffer.

[0365] Embodiment 302: The kit according to any one of embodiments 299-301, wherein said kit comprises a container containing an elution buffer.

[0366] Embodiment 303: The kit according to any one of embodiments 299-302, wherein said kit comprises a container containing a wash buffer.

[0367] Embodiment 304: The kit according to any one of embodiments 299-303, wherein said kit comprises a container containing a lysis solution according to any one of embodiments 169-171, and/or a container containing a lysis solution according to any one of embodiments 172-174.

[0368] Embodiment 305: The kit according to any one of embodiments 299-304, wherein said kit comprises a cartridge according to any one of embodiments 143-155 and/or a cartridge according to any one of embodiments 155-166.

[0369] Embodiment 306: The kit according to any one of embodiments 299-305, said kit comprising instructional materials teaching the use of said kit to convert a nuclei acid for determination of the methylation state of said nucleic acid.

[0370] Embodiment 307: A set of primers and probes for the determination of methylation of *MGMT* using a nested PCR reaction, said set comprising the following primers and probes:

[0371] an external forward primer comprising the nucleotide sequence GTTTT(T*)AGAAYG(T*)TTTGYGTTT (SEQ ID NO:263);

[0372] an external reverse primer comprising the nucleotide sequence AAAAAAC(T*)CCRCACTCTTCC (SEQ ID NO:265);

[0373] an internal forward primer comprising the nucleotide sequence TTTCGACGTTCGTAGGTTTTCGC (SEQ ID NO:266);

[0374] an internal reverse primer comprising the nucleotide sequence GCACTCTTCCGAAAACGAAACG (SEQ ID NO:267); and

[0375] a probe comprising the nucleotide sequence fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-blocker (SEQ ID NO: 268).

[0376] Embodiment 308: A set of primers and probes for the determination of methylation of *ACTB* (*e.g.*, as a control) using a nested PCR reaction, said set comprising the following primers and probes:

[0377] an external forward primer (102) comprising the nucleotide sequence GTGATGGAGGAGGTTTAGTAAGTT (SEQ ID NO:103);

[0378] an external reverse primer (103) comprising the nucleotide sequence

CCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO:104);

[0379] an internal forward primer (148) comprising the nucleotide sequence GGTTTAGTAAGTTTTTGGATTGTG (SEQ ID NO:149);

[0380] an internal reverse primer (149) comprising the nucleotide sequence CCTTAAAAATTACAAAACCACAAC (SEQ ID NO:150); and

[0381] a probe (178) comprising the nucleotide sequence fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-blocker (SEQ ID NO:179).

[0382] Embodiment 309: A set of primers and probes for the determination of methylation of *MGMT* using a nested PCR reaction with determination of the methylation of ACTB as a control, comprising the primers and probes of embodiment 307 and the primers and probes of embodiment 308.

[0383] Embodiment 310: A method of determining the methylation of *MGMT* using methylation specific PCR said method comprising:

[0384] providing a converted (*e.g.*, bisulfite converted) DNA containing a promoter region of the *MGMT* gene;

[0385] performing methylation specific PCR for *MGMT* methylation using a nested PCR reaction comprising the following primers and probes:

[0386] an external forward primer comprising the nucleotide sequence GTT TT(T*)AGAAYG(T*)TTTGYGTTT(SEQ ID NO:263);

[0387] an external reverse primer comprising the nucleotide sequence AAAAAAC(T*)CCRCACTCTTCC (SEQ ID NO:265);

[0388] an internal forward primer comprising the nucleotide sequence TTTCGACGTTCGTAGGTTTTCGC (SEQ ID NO:266);

[0389] an internal reverse primer comprising the nucleotide sequence GCACTCTTCCGAAAACGAAACG (SEQ ID NO:267); and

[0390] a probe comprising the nucleotide sequence fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-blocker (SEQ ID NO: 268); and

[0391] detecting and/or quantifying the PCR amplification product to provide determine methylation of said *MGMT* gene.

[0392] Embodiment 311: The method of embodiment 310, wherein said method further comprises:

[0393] providing a converted (*e.g.*, bisulfite converted) DNA containing a promoter region of the *ACTB* gene (*e.g.*, as a control);

[0394] performing methylation specific PCR for *ACTB* methylation using a nested PCR reaction comprising the following primers and probes:

[0395] an external forward primer comprising the nucleotide sequence GTGATGGAGGAGGTTTAGTAAGTT (SEQ ID NO:103);

[0396] an external reverse primer comprising the nucleotide sequence CCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO:104);

[0397] an internal forward primer comprising the nucleotide sequence GGTTTAGTAAGTTTTTTGGATTGTG (SEQ ID NO:149);

[0398] an internal reverse primer comprising the nucleotide sequence CCTTAAAAATTACAAAACCACAAC (SEQ ID NO:150); and

[0399] a probe comprising the nucleotide sequence fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-blocker (SEQ ID NO:179); and

[0400] detecting and/or quantifying the PCR amplification product to provide determine methylation of said *ACTB* gene.

[0401] Embodiment 312: The method according to any one of embodiments 310-311, wherein said methylation specific PCR for *MGMT* methylation and said methylation specific PCR for *ACTB* methylation are performed in a single multiplex PCR reaction.

[0402] Embodiment 313: The method according to any one of embodiments 310-312, wherein said methylation specific PCR is performed using a cartridge according to any one of embodiments 92-132.

[0403] Embodiment 314: The method of embodiment 313, wherein: said providing a converted DNA containing a promoter region of the *MGMT* gene comprises introducing an unconverted DNA containing a promoter region of the *MGMT* gene into said cartridge and operating said cartridge to convert said DNA in said cartridge using a conversion reagent; and/or said providing a converted DNA containing a promoter region of the *ACTB* gene comprises introducing an unconverted DNA containing a promoter region of the *ACTB* gene into said cartridge and operating said cartridge to convert said DNA in said cartridge using a conversion reagent.

[0404] Embodiment 315: The method of embodiment 314, wherein said conversion reagent comprises a compound selected from the group consisting of ammonium bisulfite, sodium metabisulfite, potassium bisulfite, cesium bisulfite, and DABSO.

[0405] Embodiment 316: The method according to any one of embodiments 313-315, wherein said operating said cartridge comprises heating said DNA and said conversion reagent in a thermocycling channel or chamber that is later used to perform said nested PCR reaction.

[0406] In certain embodiments the methods and/or cartridges expressly exclude magnetic materials including magnetic glass, magnetic hydroxyapatite, and magnetic matrix materials. In certain embodiments the methods and/or cartridges expressly exclude magnetic materials for DNA isolation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0407] Figure 1A illustrates major components of a cartridge (*e.g.*, a GENEXPERT® cartridge) suitable for use with the methods described herein. Fig. 1B shows a top view of the cartridge illustrating chambers disposed around a central valve. Fig. 1C shows an illustrative workflow for the determination of DNA methylation utilizing the reaction cartridge.

[0408] Figure 2, panels A-C, illustrates one embodiment of a GENEXPERT® cartridge suitable for the determination of DNA methylation as described herein.

[0409] Figures 3A-3C show illustrative, but non-limiting embodiments of the modules, and systems (*e.g.*, processing units) for the determination of DNA methylation. Fig. 3A illustrates a module for operation of a GENEXPERT® cartridge. Fig. 3B illustrates some components of one embodiment of a module for operation of a cartridge for the analysis of DNA methylation. Fig. 3C illustrates a system (*e.g.*, processing unit) incorporating a plurality of modules.

[0410] Figures 4A-4D illustrate various strategies for the use of MethyLight protocols to detect/quantify DNA phosphorylation. Fig. 4A, modified from Eads *et al.* 92000) *Nucleic Acids. Res.*, 28(8): e32) schematically illustrates MethyLight technology. DNA is modified by sodium bisulfite which generates methylation-dependent sequence differences, *e.g.*, at CpG dinucleotides by converting unmethylated cytosine residues (locations indicated by white circles) to uracil, while methylated cytosine residues (locations indicated by black circles) are retained as cytosine. Fluorescence-based PCR is then performed with primers that either overlap methylation sites or that do not overlap any methylation sites. Sequence discrimination can occur either at the level of the PCR amplification process (panel D) or at the level of the probe hybridization process (panel B), or both (panel D). Sequence discrimination at the PCR amplification level utilizes primers and probes (panel D), or just primers (panel C), to overlap potential methylation sites (*e.g.*, CpG dinucleotides). Only two (fully methylated (M) and fully unmethylated (U)) of the many theoretical methylation permutations are shown. The MethyLight assay can also be designed such that sequence discrimination does not occur at the PCR amplification level. If neither the primers nor the probe overlap sites of methylation (*e.g.*, CpG dinucleotides) (panel A), then no methylation-dependent sequence discrimination occurs at the PCR amplification or probe hybridization level. This reaction represents amplification of the converted genomic DNA without bias to methylation status, which can serve as a control for the amount of input DNA. When just the probe overlaps methylation sites (panel B), then sequence discrimination can occur through probe hybridization. Fig. 4B illustrates a MethyLight approach using a single, *e.g.*, methylation-specific, probe (PR3) along with methylation specific forward (FW) and reverse (RV) primers. Fig. 4C illustrates a MethyLight approach using multiple probes (PR1. . . PR5) that each target different regions. Fig. 4D illustrates a MethyLight approach using multiple probes (PR1. . . PR5) that each target the same region, but provide signals for different methylation patterns.

[0411] Figures 5 illustrates results from a representative GeneXpert run from 300 ng of HGDNA showing an ACTB qPCR curve and an HMBS qPCR curve.

[0412] Figure 6A and 6B illustrate the results of a titration for bisulfite-converted *ACTB* using human genomic DNA (hgDNA) in a 15 cycle nested qPCR (Fig. 6A) and a 20 cycle nested qPCR (Fig. 6B).

[0413] Figures 7A-7C shows the result of 20 cycles of nested qPCR (in the cartridge) for six methylated targets (AKR1B1, HOXB4, TM6SF1, RAASGRF2, and RASSF1A). Fig. 7A shows the results for 25 ng of HSDNA or 5000 cells without bisulfite conversion. Fig. 7B shows the results of 20 cycles of nested qPCR for the bisulfite converted methylated targets using DNA from MBA-453 cells. Fig. 7C shows the results of 20 cycles of nested qPCR for the bisulfite converted methylated targets using DNA from MBA-453 cells in a carrier (1 μg of SS and 10 ng of HS DNA). Fallouts occur at around 25-50 copies or around 100 cells.

[0414] Figure 8 illustrates the results of a determination of conversion efficiency. The conversion efficiency is about 66% (~1 Ct) the difference between unconverted HMBS and converted ACTB.

[0415] Figure 9 illustrates the increase in specificity for converted DNA produced by nested qPCR.

[0416] Figure 10 illustrates the specificity of the methylation cartridge. There is no priming off of unconverted DNA (top panel) or unmethylated DNA (bottom panel) except for HIST1H3C.

[0417] Figures 11 shows illustrative but non-limiting workflows for analysis of methylation using a cartridge (*e.g.*, a GENEXPERT® cartridge). Top illustrates one work flow for analysis of DNA methylation in a serum or plasma sample. Bottom illustrates one work flow for analysis of DNA methylation in a tissue section (*e.g.*, frozen or formalin-fixed paraffin embedded (FFPE) section).

[0418] Figure 12 illustrates the results for a FFPE cell button for converted ALU (Blue) and methylated RASSF1A (Gray).

[0419] Figure 13A illustrates a cartridge layout and Figure 13B illustrates a flow chart of the protocol used in Example 4.

[0420] Figure 14 illustrates a run in which some samples contain bisulfite contamination.

[0421] Figure 15A illustrates the results of 1000 MBA-453 cells with bisulfite conversion. Figure. 15B illustrates results of 25 ng of HS DNA control.

[0422] Figure 16 illustrates the structure of DABSO (1,4-diazoniabicyclo[2.2.2]octane-1,4-disulfinate).

[0423] Figure 17 illustrates one embodiment of a cfDNA sample preparation cartridge. The cartridge is effective for both DNA and RNA isolation. The cartridge provides three GTC-ethanol washes (GTC-ethanol washes are typically 1.25M guanidinium thiocyanate, 25 mM Tris pH 7.0, 50% ethanol), a PEG200 rinse, and a 15 mM KOH elution.

[0424] Figure 18 illustrates controls for cfDNA extraction.

[0425] Figure 19A shows a comparison of cfDNA preparation using a sample preparation cartridge as described herein compared to a standard tube-fill (*i.e.* tube-based kit) preparation. The cartridge preparation yield is very comparable to that obtained using a tube fill method. Figure 19B shows a comparison of the amount of extracted DNA detected using a cartridge-based DNA cleanup as compared to a standard tube-fill as a function of DNA amount. The cartridge-based method is conservatively within 1 Ct of the tube-fill methods and is believed to be closer at higher DNA concentration.

[0426] Figure 20A illustrates one embodiment of a high-volume (*e.g.*, up to 12 ml) sample preparation (HVSP) cartridge that can be used with a qPCR cartridge and/or with a methylation detection cartridge. Figure 20B schematically illustrates one variation of work flows in the HVSP cartridge when used in combination with a qPCR cartridge to perform a methylation analysis.

[0427] Figure 21 illustrates the detection of HBMS or β-globin using a two cartridge cleanup using a high-volume sample preparation cartridge (*see, e.g.*, Fig. 20) where the sample is transferred from the high volume cartridge to the PCR analysis cartridge compared to detection using a sample applied to a single PCR analysis cartridge resulting in less sample volume.

[0428] Figure 22 illustrates the results of bisulfite conversion using multiple heating steps (bottom panel) compared to a single heating step (top panel).

[0429] Figure 23A illustrates the steps and labor time for a methylation analysis using a standard Qiagen DNA purification kit combined with a Zymo DNA methylation kit (right) compared to a methylation analysis using a Methylation analysis cartridge described herein. Figure 23B shows a comparison of the results obtained using the two different protocols.

[0430] Figure 24 shows a comparison of DNA conversion using DABSO as the conversion reagent compared to DNA conversion using the Zymo bisulfite conversion reagent.

[0431] Figure 25, panels A and B, illustrates sensitivity of detection of methylated DNA. Panel A shows a dilution series of methylated DNA (MGMT). Panel B illustrates the sensitivity of detection of methylated pancreatic cancer markers.

[0432] Figure 26 illustrates the results for a reverse complement multiplex assay for both strands.

[0433] Figure 27A illustrates the detection of both methylated DNA and mutations in the same cartridge. Top panel illustrates detection of methylated DNA and a Kras G12D mutation in one cartridge, while bottom panel illustrates detection of methylated DNA and wildtype Kras in one cartridge. Figure 27B illustrates detection of methylated DNA and mutations I the same cartridge in two pancreatic cancer cell lines: PANC-1 cells (top panel) and MIA-PaCa cells (bottom panel).

[0434] Figure 28 illustrates temperature optimization for multiplex methylation analysis of *ADAMTS1*, and *BNC1* of a forward strand (top) and a reverse strand (bottom) of bisulfite-converted DNA.

[0435] Figure 29 illustrates the ability to multiplex the MSP primer and probe sets for *BNC1*, *ADAMTS1*, and a control gene ACTB. Probes were combined into two sets based on preferred conditions..

[0436] Figure 30 illustrates one set of primers and probes used for detection of *MGMT* methylation. Internal fwd 22150 (SEQ ID NO: 266); External fwd 22422 (SEQ ID NO: 263); Probe 22419 (SEQ ID NO: 268), Internal rev 22151 (SEQ ID NO: 267); external rev 22423 (SEQ ID NO: 265); template (SEQ ID NO: 1).

[0437] Figure 31 shows the results of a comparison between bisulfite pyrsequencing and a MGMT methylation cartridge for extracted DNA (top) and for an FFPET sample (bottom).

[0438] Figure 32 illustrates *BRCA1* primer and probe set optimization of ΔCt between methylated converted and unmethylated converted DNA.

[0439] Figure 33 illustrates a one target assay for BRCA1 methylation tested with the ACTB control gene. As shown, eight different cell lines were tested and the effect of adding NH_4 was compared.

[0440] Figure 34 illustrates the results of a three target methylation assay for genes whose methylation is associated with lung cancer (*SOX17, CD01, TAC1*) in a background of normal plasma and in in three different lung cancer cell lines.

<u>Definitions</u>

[0441] To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

[0442] As used herein, the terms "detect", "detecting" or "detection" may describe either the general act of discovering or discerning or the specific observation of a detectably labeled composition.

[0443] As used herein, the term "detectably different" or "spectrally distinguishable" refers to a set of labels (such as dyes/fluorophores) that can be detected and distinguished simultaneously.

[0444] DNA methylation DNA methylation refers to the addition of a methyl group (CH_3) covalently to the base cytosine (C) typically in the dinucleotide 5′-CpG-3′. The term CpG refers to the base cytosine (C) linked by a phosphate bond to the base guanine (G) in the DNA nucleotide sequence.

[0445] The term "conversion reagent" refers to a reagent that deaminates cytosine to uracil in single stranded DNA, while leaving 5-MeC essentially unaffected. Illustrative conversion reagents include bisulfites (*e.g.*, sodium metabisulfite, potassium bisulfite, cesium bisulfite, ammonium bisulfite, *etc.*) and/or compounds that can produce a bisulfite under appropriate reaction conditions (*e.g.*, DABSO).

[0446] The phrase "detecting methylation of a gene" generally refers to the detection of methylation of cytosine, typically in CPG islands, in the promoter region of the gene.

[0447] As used herein, the terms "patient" and "subject" are typically used interchangeably to refer to a human. In some embodiments, the methods described herein may be used on samples from non-human animals, *e.g.*, a non-human primate, canine, equine, feline, porcine, bovine, lagomorph, and the like.

[0448] As used herein, the terms "oligonucleotide," "polynucleotide," "nucleic acid molecule," and the like, refer to nucleic acid-containing molecules, including but not limited to, DNA. The terms encompass sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyamino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

[0449] As used herein, the term "oligonucleotide," refers to a single-stranded polynucleotide typically having fewer than 500 nucleotides. In some embodiments, an oligonucleotide is 8 to 200, 8 to 100, 12 to 200, 12 to 100, 12 to 75, or 12 to 50 nucleotides long. Oligonucleotides may be referred to by their length, for example, a 24 residue oligonucleotide may be referred to as a "24-mer."

[0450] As used herein, the term "complementary" to a target gene (or target region thereof), and the percentage of "complementarity" of the probe sequence to the target gene sequence is the percentage "identity" to the sequence of target gene or to the complement of the sequence of the target gene. In determining the degree of "complementarity" between probes used in the compositions described herein (or regions thereof) and a target gene, such as those disclosed herein, the degree of "complementarity" is expressed as the percentage identity between the sequence of the probe (or region thereof) and sequence of the target gene or the complement of the sequence of the target gene that best aligns therewith. The percentage is calculated by counting the number of aligned bases that are identical as between the 2 sequences, dividing by the total number of contiguous nucleotides in the probe, and multiplying by 100. When the term "complementary" is used, the subject oligonucleotide is at least 90% complementary to the target molecule, unless indicated otherwise. In some embodiments, the subject oligonucleotide is at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary to the target molecule.

[0451] A "primer" or "probe" as used herein, refers to an oligonucleotide that comprises a region that is complementary to a sequence of at least 8 contiguous nucleotides of a target nucleic acid molecule, such as a target gene. In some embodiments, a primer or probe comprises a region that is complementary to a sequence of at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 29, at least 30, at least 319, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, or at least 40 contiguous nucleotides of a target molecule. When a primer or probe comprises a region that is "complementary to at least x contiguous nucleotides of a target molecule," the primer or probe is at least 95% complementary to at least x contiguous nucleotides of the target molecule. In some embodiments, the primer or probe is at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary to the target molecule.

[0452] The term "nucleic acid amplification," encompasses any means by which at least a part of at least one target nucleic acid is reproduced, typically in a template-dependent manner, including without limitation, a broad range of techniques for amplifying nucleic acid sequences, either linearly or exponentially. Exemplary means for performing an amplifying step include polymerase chain reaction (PCR), ligase chain reaction (LCR), ligase detection reaction (LDR), multiplex ligation-dependent probe amplification (MLPA), ligation followed by Q-replicase amplification, primer extension, strand displacement amplification (SDA), hyperbranched strand displacement amplification, multiple displacement amplification (MDA), nucleic acid strand-based amplification (NASBA), two-step multiplexed amplifications, rolling circle amplification (RCA), and the like, including multiplex versions and combinations thereof, for example but not limited to, OLA/PCR, PCR/OLA, LDR/PCR, PCR/PCR/LDR, PCR/LDR, LCR/PCR, PCR/LCR (also known as combined chain reaction--CCR), digital amplification, and the like. Descriptions of such techniques can be found in, among other sources, Ausbel *et al.*; PCR Primer: A Laboratory Manual, Diffenbach, Ed., Cold Spring Harbor Press (1995); The Electronic Protocol Book, Chang Bioscience (2002); Msuih *et al.*, J. Clin. Micro. 34:501-07 (1996); The Nucleic Acid Protocols Handbook, R. Rapley, ed., Humana Press, Totowa, N.J. (2002); Abramson *et al.*, Curr Opin Biotechnol. 1993 February; 4(1):41-7, U.S. Pat. No. 6,027,998; U.S. Pat. No. 6,605,451, Barany *et al.*, PCT Publication No. WO 97/31256; Wenz *et al.*, PCT Publication No. WO 01/92579; Day *et al.*, Genomics, 29(1): 152-162 (1995), Ehrlich *et al.*, Science 252:1643-50 (1991); Innis *et al.*, PCR Protocols: A Guide to Methods and Applications, Academic Press (1990); Favis *et al.*, Nature Biotechnology 18:561-64 (2000); and Rabenau *et al.*, Infection 28:97-102 (2000); Belgrader, Barany, and Lubin, Development of a Multiplex Ligation Detection Reaction DNA Typing Assay, Sixth International Symposium on Human Identification, 1995 (available on the world wide web at: promega.com/geneticidproc/ussymp6proc/blegrad.html); LCR Kit Instruction Manual, Cat. #200520, Rev. #050002, Stratagene, 2002; Barany, Proc. Natl. Acad. Sci. USA 88:188-93 (1991); Bi and Sambrook, Nucl. Acids Res. 25:2924-2951 (1997); Zirvi *et al.*, Nucl. Acid Res. 27:e40i-viii (1999); Dean *et al.*, Proc Natl Acad Sci USA 99:5261-66 (2002); Barany and Gelfand, Gene 109:1-11 (1991); Walker *et al.*, Nucl. Acid Res. 20:1691-96 (1992); Polstra *et al.*, BMC Inf. Dis. 2:18-(2002); Lage *et al.*, Genome Res. 2003 February; 13(2):294-307, and Landegren *et al.*, Science 241:1077-80 (1988), Demidov, V., Expert Rev Mol Diagn. 2002 November; 2(6):542-8., Cook *et al.*, J Microbiol Methods. 2003 May; 53(2):165-74, Schweitzer *et al.*, Curr Opin Biotechnol. 2001 February; 12(1):21-7, U.S. Pat. No. 5,830,711, U.S. Pat. No. 6,027,889, U.S. Pat. No. 5,686,243, PCT Publication No. W00056927A3, and PCT Publication No. W09803673A1.

[0453] In some embodiments, amplification comprises at least one cycle of the sequential procedures of: annealing at least one primer with complementary or substantially complementary sequences in at least one target nucleic acid; synthesizing at least one strand of nucleotides in a template-dependent manner using a polymerase; and denaturing the newly-formed nucleic acid duplex to separate the strands. The cycle may or may not be repeated. Amplification can comprise thermocycling or, in certain embodiments, can be performed isothermally.

[0454] The term "hybridize" is typically used herein refer to "specific hybridization" which is the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence, in some embodiments, under stringent conditions. The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target sequence, and to a lesser extent to, or not at all to, other sequences. A "stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization are sequence-dependent and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in, *e.g.*, Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I, Ch. 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, N.Y. ("Tijssen"). Generally, highly stringent hybridization and wash conditions for filter hybridizations are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. In certain embodiments very stringent conditions are selected to be equal to the T_m for a particular probe. Dependency of hybridization stringency on buffer composition, temperature, and probe length are well known to those of skill in the art (see, *e.g.*, Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual* (3rd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY).

[0455] A "sample," as used herein, generally refers to a biological sample including biological fluids (*e.g.*, blood or blood fractions, serum, plasma, pancreatic juice, cerebrospinal fluid, oral fluid, lymph, intraocular fluid, and the like) and/or tissue samples including, but not limited to biopsy samples, frozen tissue samples, formalin fixed paraffin embedded (FFPE) samples from various tissues including but not limited to breast tissue, endocervical tissue, vaginal tissue,colon/rectal tissue, throat tissue, and other types of human samples, such as blood, stool, and biopsy samples. The term sample also includes diluted and/or buffered forms of the above samples, for example, a buffer into which a swab sample has been placed, a urine sample to which a buffer has been added, and the like.

[0456] As used herein, the phrase "is indicative of the presence of a cancer or a predisposition to a cancer" means that a particular result tends to indicate that a cancer is present, and/or a precancerous condition is present or likely present. This phrase does not imply a definitive determination that the condition is present. A definitive determination can be made based on further examination or testing that a medical practitioner deems appropriate. Furthermore, this phrase does not require that a determination be made as to which condition may be present based only on the particular result. Rather, it is contemplated that a positive result will be considered in light of other examination or text results to arrive at a differential diagnosis.

[0457] The term "tubefill procedure" refers to a procedure that is run using standard laboratory instrumentation rather than on a cassette (*e.g.*, rather than with a GENEXPERT®, or modified GENEXPERT® cartridge described herein).

DETAILED DESCRIPTION

[0458] In various embodiments devices and methods are provided that facilitate the rapid detection and/or characterization of methylation in DNA samples. In certain embodiments automated reaction cartridges are provided as are methods that that utilize the automated reaction cartridge(s) to facilitate analysis of the methylation of a DNA sample and, optionally, to measure mRNA levels along with the determination of DNA methylation. In various embodiments the DNA methylation is determined by bisulfite conversion and analysis of the bisulfite converted DNA (*e.g.*, via methylation specific PCR, nucleic acid sequencing, melting point analysis, and the like). In certain embodiments the cartridge performs all or a part of the bisulfite conversion of DNA and all or a part of the analysis of the bisulfite converted DNA. In certain embodiments the cartridge performs all of the steps involved in bisulfite conversion and all or a part of the analysis of the bisulfite-converted DNA. In certain embodiments the cartridge performs all of the steps involved in bisulfite conversion and all of the analysis of the bisulfite-converted DNA. In certain embodiments the cartridge additionally performs an isolation and purification of the DNA to be analyzed.

[0459] There are several advantages to automating the methylation analysis including for example, reduction in overall processing time, improvements in efficiency, decreased user error and variability, minimization of loss between steps, and an improved ability to use smaller amounts of sample. Use of a cartridge-based process, as described herein, allows for rapid and easy testing of not only multiple sample types but also for evaluating methylation changes observed in several different types of cancers including, but not limited to breast cancer, colorectal cancer, prostate cancer, and lung cancer.

[0460] The cartridge-based methods described herein additionally permit measurement of mRNA derived from the same sample. Measurement of corresponding upstream and/or downstream mRNA involved in DNA methylation can be important to understand the mechanism and activity of the epigenetic modification. For example, the measurement of DNA methyltransferases (DNMT) mRNA has been studied along with DNA methylation for several cancers (*see* Table 1).

Table 1. Illustrative DNA methyltransferases and their importance in particular cancers (from Subramaniam *et al.* (2014) *Front Oncol.*, 4: Article 80, doi: 10.3389/fonc.2014.00080).

Methyltransferase	Cancers
DNMT1	Leukemia: upregulated - 5.3-fold expression Gastric cancer - 64.8% localized in the cytoplasm and nuclei Breast cancer - 16.6% Hepatocellular carcinoma - 100% Pancreatic cancer - highly expressed - Gli target gene Colon cancer - highly expressed Glioblastoma - overexpressed
DNMT2 or TRDMT1	Hepatocellular carcinoma - reduced expression Colorectal and stomach cancers - lower mRNA expression
DNMT3A	Acute myeloid leukemia - 22.1 % mutations and affect translation Gastric cancer - 70,4% localized in the cytoplasm Breast cancer - 14% Hepatocellular carcinoma - 60% Pancreatic cancer - highly expressed - regulated by Gli 1 Colon cancer - highly expressed
DNMT3B	Leukemia: upregulated- 11.7-fold expression Gastric cancer - 51.9% localized in the cytoplasm Breast cancer - 81.8% poor prognosis Breast cancer cell lines-hypermethylation defect resulted in aberrant - overexpression DNMT activity Hepatocellular carcinoma (60%) and mRNA levels high Colon cancer - highly expressed Prostate cancer - overexpressed Glioblastoma - overexpressed

DNMT3L	Cervical cancer - promising biomarker Embryonal carcinoma - novel biomarker

[0461] Often separate independent extractions for DNA or RNA are used for studying and measuring genes and transcripts. Co-detection from the same sample preparation would be ideal to minimize sample preparation, assay to assay, sample-to-sample and cell-to-cell variability.

Cartridge-based bisulfite conversion of DNA

[0462] In certain embodiments the extraction of DNA, bisulfite conversion, and methylation specific PCR are all performed in the cartridge. In one illustrative embodiment, the user will add the sample to a lysis/binding reagent, then mix/vortex the reagent briefly, and then add the sample to a sample port or chamber in the cartridge. Illustrative, but non-limiting lysis reagents (including reagents particularly well suited for FFPE sections) are described in PCT Patent Publication No: WO/2014/052551 (PCT/US2013/061863), which is incorporated herein by reference for the reagents described therein.

[0463] Additional illustrative lysis reagents for serum or plasma and for formalin-fixed paraffin embedded (FFPE) samples are shown in Example (Tables 11, and 12, respectively).

[0464] In certain embodiments the cartridge is placed into a processing module and the assay is initiated by clicking through a set of selections within software controlling the processing module (*see, e.g.*, Figs. 11A and 11B). The cartridge then performs the bisulfite conversion process and analysis of the bisulfite-converted DNA. In certain embodiments mRNA is also determined. While in certain embodiments, all of the operations are performed in the cartridge, in other embodiments, subsets of the various operations are performed in the cartridge as described below.

[0465] The sample can comprise any biological sample that contains DNA whose methylation state is to be evaluated. Illustrative samples include, but are not limited to isolated DNA and/or isolated total nucleic acids, a cell, a tissue, a biological fluid containing a nucleic acid, and the like. In certain embodiments the biological sample comprises a biological fluid selected from the group consisting of plasma, serum, amniotic fluid saliva, mucus, urine, pancreatic juice, and cerebrospinal fluid. In certain embodiments the sample comprises a tissue sample from a healthy tissue, or a tissue sample from a diseased sample. In certain embodiments the tissue sample is from a fetus, a neonate, a child, an adolescent, or an adult. In certain embodiments the tissue sample comprises tumor cell and/or is derived from a biopsy of a tumor (*e.g.*, a breast cancer, a prostate cancer, a brain cancer, a cervical cancer, an ovarian cancer, a pancreatic cancer, a colon cancer, a gastric cancer, a hepatocellular cancer and the like. In certain embodiments the sample comprises a fixed tissue, *e.g.*, a formalin fixed tissue sample. In certain embodiments the sample comprises an embedded tissue sample (*e.g.*, a formalin-fixed paraffin embedded (FFPE) tissue sample).

[0466] Bisulfite conversion of DNA typically involves four steps:

[0467] 1) DNA purification;

[0468] 2) DNA denaturation;

[0469] 3) DNA conversion (*e.g.*, bisulfite deamination); and

[0470] 4) Alkali desulphonation.

[0471] Typically DNA conversion (*e.g.*, using a conversion reagent such as a bisulfite) involves: 1) Sulphonation: The addition of bisulphite to the 5-6 double bond of cytosine; and 2) Hydrolic Deamination: hydrolytic deamination of the resulting cytosine-bisulphite derivative to give a uracil-bisulphite derivative. This is followed by Alkali Desulphonation: Removal of the sulphonate group by an alkali treatment, to give uracil as indicated above.

[0472] As noted above, in certain embodiments, the DNA purification can be performed prior to placing a sample in the cartridge, or alternatively, can be performed by the cartridge itself. Accordingly, in certain embodiments the sample is added directly to the reaction cartridge, while in other embodiments, the sample is mixed with one or more reagents. In certain embodiments DNA preparation typically involves preparing substantially isolated DNA. This may involve lysing cells to release DNA, removing particulates and cellular debris, and/or removing protein components to provide a sample comprising substantially pure nucleic acids (*e.g.*, substantially pure DNA and/or a substantially pure combination of DNA and RNA). In one illustrative, but non-limiting, embodiment, the sample (*e.g.*, a tissue sample) is added to a lysis reagent, agitated and then inserted into the cartridge for further processing.

[0473] In certain embodiments, all of the reagents necessary to perform bisulfite conversion of the DNA are provided in the cartridge. In certain embodiments, to avoid degradation of reagents over time in the cartridge, certain reagents may be added to the cartridge immediately before use. Thus, for example in certain embodiments, it is contemplated that the cartridge may be loaded with a conversion reagent (*e.g.*, a bisulfite reagent) and/or a guanidium thiocynanate reagent (*e.g.*, GTC-EtOH-Tween) at or about the time the sample is loaded into the cartridge. In certain embodiments, the guanidinium thiocyanate reagent (*e.g.*, GTC-EtOH-Tween) is combined with the sample and added to the cartridge in the sample receiving chamber (*e.g.*, chamber 2 in the GENEXPERT® cartridge).

[0474] In certain embodiments when performing the bisulfite conversion of DNA using a reaction cartridge (*e.g.*, GENEXPERT® cartridge), the method comprises

[0475] i) contacting a biological sample comprising a nucleic acid to a first matrix material comprising a first column or filter where said matrix material binds and/or filters nucleic acids in said sample and thereby purifies the DNA;

[0476] ii) eluting the bound DNA from the first matrix material (*e.g.*, using an alkaline solution) and denaturing the DNA to produce eluted denatured DNA;

[0477] iii) heating the eluted DNA in the presence of a conversion reagent (*e.g.*, a reagent that provides bisulfite ions) to produce a converted (*e.g.*, a deaminated) nucleic acid;

[0478] iv) contacting the converted nucleic acid to a second matrix material comprising a second column to bind said deaminated nucleic acid to said second matrix material (note in certain embodiments the second column can be a column different than the first column, or in other embodiments, the same column used a second time);

[0479] v) desulfonating the bound deaminated nucleic acid and/or simultaneously eluting and desulfonating the nucleic acid by contacting the deaminated nucleic acid with an alkaline solution to produce a converted (*e.g.*, bisulfite converted) nucleic acid; and

[0480] vi) eluting the converted nucleic acid from said second matrix material, wherein at least steps iv) through vi) are performed in a one reaction cartridge.

[0481] In certain embodiments the method further includes the analysis of the converted DNA. Accordingly, in certain embodiments, the method further comprises:

[0482] vii) performing methylation specific PCR and/or nucleic acid sequencing, and/or high resolution melting analysis (HRM) on the converted nucleic acid to determine the methylation of the nucleic acid, wherein at least steps iv) through vi) are performed in a single reaction cartridge.

[0483] In certain embodiments at least steps iii) through vi) are performed in one reaction cartridge.

[0484] In certain embodiments at least steps ii) through vi) are performed in one reaction cartridge.

[0485] In certain embodiments at least steps i) through vi) are performed in one reaction cartridge.

[0486] In certain embodiments at least steps i) through vii) are performed in one reaction cartridge.

[0487] It is noted that the first column and, where present, the second column can refer to discrete columns. However, particularly when integrated into a reaction cartridge, the "column" can simply be a matrix material disposed in a chamber or channel in the cartridge. In various embodiments the "columns" act as filters and/or as affinity columns that bind nucleic acids. Accordingly, in certain embodiments the column contains a matrix material that binds a nucleic acid (*e.g.*, DNA and/or RNA). Illustrative matrix materials include, but are not limited to, glass (silica), an ion exchange resin, hydroxyapatite, and the like. It will be recognized that the matrix materials can take a number of forms. Thus, in certain embodiments, the matrix material comprises a fibrous material a particulate material (*e.g.*, microbeads, nanobeads, *etc.*), a structured material (*e.g.*, porous "baffle" system", a serpentine channel, and the like). In certain embodiments the first column and second column are different columns (chambers or channels). In other embodiments the first column and the second column are the same column (chamber or channel) that is used twice (*e.g.*, a first time and a second time).

[0488] In certain embodiments, the use of one or more additional filters, *e.g.*, to clean up the initial sample prior to contacting with the first matrix material, is contemplated. Thus, for example, in certain embodiments, a filter matrix (*e.g.*, polycarbonate filter, nylon filter, polypropylene filter, polyester filter, nylon filter, ceramic filter, polytetrafluoroethylene filter, and the like) is disposed in the sample receiving chamber or "downstream" from the sample receiving chamber and before the first "column". It is also recognized, that in certain embodiments, the sample, can be lysed and/or filtered prior to deposition into a sample receiving chamber.

[0489] In certain illustrative ,but non-limiting embodiments, the methods described herein can be performed using a GENEXPERT® cartridge (Cepheid, Inc., Sunnyvale, CA) or a variant thereof. In various embodiments sample extraction, and/or amplification, and/or DNA conversion, and/or detection can all be carried out within this self-contained "laboratory in a cartridge" (*see, e.g.*,., U.S. Pat. Nos. 5,958,349, 6,403,037, 6,440,725, 6,783,736, and 6,818,185, each of which is herein incorporated by reference in its entirety). In various embodiments components of the cartridge can include, but are not limited to, processing chambers containing reagents, filters, and capture technologies useful to extract, purify, and amplify target nucleic acids. A valve enables fluid transfer from chamber to chamber and contains nucleic acids lysis and filtration components. An optical window enables real-time optical detection (*e.g.*, of PCR amplification products). A reaction tube can be provided that permits very rapid heating and/or thermal cycling.

[0490] In certain embodiments an illustrative GENEXPERT® cartridge comprises a plurality of chambers disposed around a central valve assembly and selectively in fluid communication with the central valve assembly where the central valve assembly is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with the central valve. Rotation of the valve assembly determines which chamber are in fluid communication with the central valve. One illustrative GENEXPERT® cartridge is illustrated in Figure 1A which show the cartridge, processing/reagent chambers, a reaction tube (*e.g.*, heating and/or thermocycling tube), optional optical windows, and a valve that facilitates fluid transfer from chamber to chamber.

[0491] An illustrative layout of the cartridge is shown in Figure 1B which provides a top view of the cartridge identifying various chambers by number. In one illustrative, but non-limiting embodiment, the components of the chambers comprising the cartridge are as listed in Table 2. It will be recognized that this disposition of reagents and chamber is illustrative and non-limiting. Using the teachings provided herein other reagent dispositions and/or other chamber configurations will be available to one of skill in the art.

Table 2. One illustrative embodiment showing chamber contents for use of a GENEXPERT® cartridge for measurement of DNA methylation.

Chamber #	Chamber Contents	Initial Volume (µL)
1		--
2*	Sample chamber (sample mixed with *e.g.*, GTC-EtOH-Tween precipitation reagent)	
3**	GTC-EtOH	

4* **	Bisulfite reagent (*e.g.*, 8M ammonium bisulfite)	
5	Buffer *e.g.*, 50 mM Tris pH 8.5	
6		--
7		--
8	Rinse (*e.g.*, PEG 200)	
9	Beads (*e.g., polymerase, primer, probe)*	
10	Elution/Desulphonation reagent (*e.g.*, 15 mM KOH)	
11	Beads (*e.g., polymerase, primer, probe)*	
*Sample is added to chamber 2 by user **In certain embodiments, GTC-EtOH is added at time of use (*e.g.*, when sample is added). In certain embodiments GTC-EtOH is provided as reagent already disposed in cartridge. ***In certain embodiments, bisulfite reagent is added at time of use (*e.g.*, when sample is added). In certain embodiments bisulfite reagent is already disposed in cartridge.		

[0492] One embodiment of a step-by-step workflow for the determination of DNA methylation utilizing such a cartridge is shown in Figure 1C. In this cartridge configuration, there are five chambers that, in use (*e.g.*, when the cartridge is operating to determine DNA methylation), will hold reagents and buffers (*e.g.*, chambers 3, 4, 5, 8, and 10), one chamber that will hold the sample added by the user (*e.g.*, chamber 2), and one or two (or more) chambers holding analysis reagents (*e.g.*, MSP reagents such as enzyme reaction, template specific reaction, and/or or 200 mM Tris pH 7.0, *e.g.*, as beads) (*e.g.,* chambers 9, and 11). In certain embodiments, the reagents (*e.g.*, polymerase, reverse transcriptase, primer, probe) are provided in solution. In certain embodiments the reagents are provided as lyophilized powders. In certain embodiments the reagents are provided as lyophilized beads. The beads can further comprise agents to that improve reagent stability and/or activity (*see, e.g.*, U.S. Patent Publication No: 2006/0068399 which is incorporated herein by reference for the beads, bead fabrication, and bead formulations described therein.

[0493] In certain embodiments the cartridge, as provided contains all of the reagents necessary to run the cartridge and only the sample (*e.g.*, sample in buffer/lysis/precipitation solution) is added to the cartridge. In certain embodiments the cartridge is provided without the GTC-EtOH and/or the bisulfite reagents and one or both are added at the time of use.

Thus, in certain embodiments, the GTC-EtOH reagent is added to the cartridge at the time of use, in certain embodiments the bisulfite reagent (in addition to the sample) is added to the chamber at the time of use, and in certain embodiments, both the GTC-EtOH and the bisulfite reagent (in addition to the sample) are added to the cartridge at the time of use. In certain embodiments these reagents are added directly to the desired chambers (*see, e.g.*, Table 2). In certain embodiments ports are provided for loading the reagents and the ports are configured to deliver the reagent(s) to the desired chambers.

[0494] At the start of the assay, the cartridge dispenses the sample, *e.g.* from chamber 2 over a glass fiber column (*e.g.* the first column) in the cartridge. DNA is eluted off the column and simultaneously denatured by an alkali solution, *e.g.*, a low concentration of potassium hydroxide from chamber 10 into a concentrated bisulfite reagent (*e.g.*, concentrated ammonium bisulfite) in Chamber 4. In certain embodiments the DNA is eluted with an alkaline solution of KOH with a pH greater than about 10.5, or a pH greater than about pH 12. In certain embodiments the DNA is eluted with 10-15 mM KOH.

[0495] As indicated above, the DNA is eluted (optionally with a burst of sonication) into the bisulfite reagent. In various embodiments the conversion reagent (*e.g.*, bisulfite reagent) is present at a concentration ranging from about 4 M to about 10 M, or from about 5 M to about 8 M, or from about 6 M or about 7 M. In certain embodiments the bisulfite solution comprises sodium metabisulfite, or potassium bisulfite, or ammonium bisulfite, or cesium bisulfite, or DABSO (1,4-diazoniabicyclo[2.2.2]octane-1,4-disulphinate, *see, e.g.*, Fig. 16). In certain embodiments the conversion reagent (*e.g.*, bisulfite reagent) contains radical scavengers, including, but not limited to one or more chemicals to prevent sulfite oxidation to sulfate (TROLOX and hydroquinone), and/or catalysts (polyamines).

[0496] The DNA-bisulfite (DNA/conversion reagent) mix is then introduced into a temperature controlled chamber or channel and incubated at a temperature ranging from about 40°C to about 95°C. In certain embodiments the mix is incubated at a constant temperature, while in other embodiments, *e.g.*, where the temperature controlled chamber or channel is a thermocycling chamber or channel (*e.g.*, a smartcycler tube in the back of the cartridge), the mix is thermally cycled (*e.g.*, between 60°C and 95°C). The mix is incubated until the DNA is converted (*e.g.*, deaminated). In certain embodiments the incubation is for a period of time that ranges from about 5 minutes up to about 4 hours, or preferably from about 15 minutes up to about 45 minutes.

[0497] Following incubation the DNA/conversion reagent) (*e.g.*, DNA-bisulfite) solution is mixed with fresh guanidinium thiocyanate-EtOH, *e.g.*, from chamber 3 and dispensed over a matrix material. In certain embodiments the first column is reused, hence there is only one column and the second column and the first column are the same. In certain embodiments the second column is a separate column different than the first column.

[0498] The DNA bound to the second column matrix material is washed with fresh GTC-EtOH (*e.g.*, from chamber 3) and rinsed (*e.g.*, with a PEG 200 rinse, *e.g.*, from chamber 8). The DNA is then desulfonated on the column, or is simultaneously eluted and desulfonated by contacting the deaminated nucleic acid with an alkaline solution (*e.g.*, KOH from chamber 10 to produce a bisulfite converted nucleic acid. In certain embodiments the incubation is for a period of time ranging from about 1 minute to about 1 hour, or from about 5 minutes to about 30 minutes, or from about 10 minutes to about 20 minutes, or for about 15 minutes.

[0499] Where the initial incubation was in a thermocycling chamber that is to be further used, the thermocycling chamber or channel is washed with a buffer to remove residual bisulfite and neutralize pH. It was a surprising discovery that incubation with a conversion reagent (*e.g.*, a bisulfite reagent), and/or desulphonation can be performed in a channel or chamber that is later used for PCR without bisulfite contamination substantially interfering with the later PCR reaction(s).

[0500] The eluted desulphonated bisulfite-converted DNA can be mixed with an appropriate buffer and analyzed for methylation. In certain embodiments the converted DNA is mixed with concentrated Tris, enzyme reaction, and template specific beads (*e.g.*, beads comprising primers and/or probes for the PCR or nested PCR reaction(s)) in chambers 9 and 11, and the final mixture is aspirated into the thermocycling tube or chamber for the methylation specific quantitative PCR reaction.

[0501] Bisulfite contamination during the qPCR step can be the primary failure mode of the methylation cartridge. Residual bisulfite can result from either direct contamination of the PCR reaction tube (*e.g.*, during the bisulfite conversion step) or from indirect contamination (*e.g.* cross contamination during bisulfite fluidic movements between chambers). Residual bisulfite contamination, if present, can be measured by bisulfite-mediated probe cleavage during the qPCR step, which results in an increase in fluorescence during the earlier qPCR cycles (cycles 1-10) typically used for background subtraction. Accordingly, in certain embodiments, the cartridge comprises beads that provide one or more probes that are cleavable during PCR if bisulfite is present. Results of a run containing bisulfite contamination are shown in Figure 14.

[0502] While the methods above (and in Example 4, *see, e.g.,* Fig. 13A) are described with respect to specific chambers in the GENEXPERT® cartridge, it will be recognized that the particular reagent/chamber assignments can be varied depending on the particularities of the methylation analysis protocol applied.

[0503] Thus, for example, operation of a methylation analysis cartridge (*e.g.*, a GENEXPERT® cartridge can be generally described by a flow chart (see, *e.g.*, Figures 1C and 13B). In the illustrative, but non-limiting embodiment shown in Fig. 13B, the DNA sample is provided in a binding buffer (*e.g.*, a buffer comprising GTC-EtoH, in certain embodiments after the sample is processed with proteinase K and/or a lysis solution). In certain embodiments the sample is obtained from a sample preparation cartridge as described herein (see, *e.g.*, Figure 20).

[0504] The sample in binding buffer is introduced into a sample receiving chamber of the cartridge. In operation the cartridge is operated to deliver the sample solution to a matrix ("column") that binds the DNA. The bound DNA is then eluted from the column using an alkaline reagent (*e.g.*, KOH solution) combined with a bisulfite reagent and moved to a heating tube (typically the PCR reaction tube) in the cartridge where the bisulfite reaction proceeds (*e.g.*, at about 50°C or about 60°C to about 90°C for about 45 minutes (or up to about 90 minutes), in this illustrative protocol). The reacted DNA is combined with a binding buffer (*e.g.*, 2.25 M Guanidinium thiocyanate, 22.5 mM Tris pH 7.0, 0.5% Tween20, 50% Ethanol, and 0.005% SE-15 antifoam (a 10% emulsion of an active silicon antifoam and non-ionic emulsifiers)) and moved back to the same column, or to a different column, where it again binds to the column matrix. The reacted DNA is washed with GTC-EtOH, rinsed with PEG (*e.g.*, PEG200) and eluted again from the column using an alkaline reagent (*e.g.*, KOH) which also desulphonates the DNA. While the DNA is desulphonating the reaction tube (*e.g.*, PCR reaction tube) can be heated and rinsed (*e.g.*, 10 x rinse) to remove any bisulfite reagent. The eluted DNA (or a portion thereof) can be moved to a reaction tube for PCR and/or nested PCR.

[0505] It will be appreciated that these operations can be performed on the entire sample or on a portion of the DNA sample. In the latter case a portion of the sample can be stored in one or more chambers and used as a control, or subjected to a different analysis/protocol.

Co-purification and detection of both RNA expression and DNA methylation.

[0506] In certain embodiments methods for co-purification and detection of both altered RNA expression of genes along with DNA methylation (MSP) in a cartridge-based assay (*e.g.*, utilizing a GENEXPERT® cartridge) are provided. In certain embodiments these assays would identify altered expression of *e.g.* DNMT correlated with tumor-specific methylation from the same sample preparation. In certain embodiments these assays can be used to verify expression and methylation status.

[0507] We have shown that we can elute nucleic acids off the column using a Tris buffered elution that retains a portion of nucleic acids on the column. In one illustrative embodiment, an RNA fraction is eluted and retained, *e.g.*, in a chamber in the cartridge using a Tris solution.

[0508] After saving the RNA fraction, NaOH or KOH elution which will strip the column and elute and denature the DNA which would go into bisulfite for conversion as described above. Then, ether using the RNA elution fraction to elute the bisulfite converted DNA from the column or using the KOH elution mix the two fractions (RNA and converted DNA products) are mixed for RNA plus bisulfite converted qRT-PCR. This involves incorporating a reverse transcriptase (RT) step for the RNA plus MSP (or other analytic method) in the same tube from the same sample. Alternative methods include, but are not limited to performing the RT step independently prior to mixing with DNA (combine cDNA and DNA) for qPCR, or PCR for DNA or RT RNA could be done independently/serially using one thermocycling tube/chamber or simultaneously using multiple thermocycling tubes/chambers in the cartridge.

Analysis of converted DNA

[0509] Numerous analytic methods can be performed in the cartridge to evaluate DNA methylation. Alternatively, in certain embodiments, the cartridge can be coupled to another device and/or system for further analysis of the converted (*e.g.*, bisulfite or DABSO converted) DNA. Illustrative methods include, but are not limited to methylation specific PCR (MSP), direct sequencing, high resolution melting analysis (HRM), pyrosequencing (sequencing by addition), base-specific cleavage analysis (*e.g.* base-specific MALDI-TOF), and the like.

Methylation-specific PCR (MSP).

[0510] In various embodiments methylation-specific PCR can be used to evaluate methylation status of the target DNA. MSP utilized primer and/or probe sets designed to be "methylated-specific" by including sequences complementing only unconverted 5-methylcytosines, or, on the converse, "unmethylated-specific", complementing thymines converted from unmethylated cytosines. Methylation is then determined by the ability of the specific primer to achieve amplification. This method is particularly effective for interrogating CpG islands in regions of high methylation density, because increased numbers of unconverted methylcytosines within the target to be amplified increase the specificity of the PCR. In certain embodiments placing the CpG pair at the 3'-end of the primer also improves the specificity.

[0511] In certain embodiments methylation is evaluated using a MethyLight method. The MethyLight method is based on MSP, but provides a quantitative analysis using quantitative PCR (*see, e.g.*, Eades *et al.* (2000) *Nucleic Acids Res.*, 28(8): E32. doi:10.1093/nar/28.8.e32). Methylated-specific primers are used, and a methylated-specific fluorescence reporter probe is also used that anneals to the amplified region. In alternative fashion, the primers or probe can be designed without methylation specificity if discrimination is needed between the CpG pairs within the involved sequences. Quantitation can be made in reference to a methylated reference DNA. One modification to this protocol to increase the specificity of the PCR for successfully bisulphite-converted DNA (ConLight-MSP) uses an additional probe to bisulphite-unconverted DNA to quantify this non-specific amplification (*see, e.g.,* Rand *et al.* (2002) *Methods* 27(2): 114-120).

[0512] In various embodiments the MethyLight methods utilize TAQMAN® technology, which is based on the cleavage of a dual-labeled fluorogenic hybridization probe by the 5' nuclease activity of Taq-polymerase during PCR amplification (Eads *et al.* (1999) *Cancer Res.*, 59: 2302-2306; Livak *et al.* (1995) *PCR Meth. Appl.*, 4: 357-362; Lee *et al.* (1993) *Nucleic Acids Res.*, 21: 3761-3766; Fink *et al.* (1998) *Nat. Med.*, 4: 1329-1333). The use of three different oligonucleotides in the TAQMAN® technology (forward and reverse PCR primers and the fluorogenic hybridization probe) offers the opportunity for several sequence detection strategies.

[0513] For example, the sequence discrimination can occur at the level of the PCR amplification process (*see, e.g.,* Fig. 4A, panel C) and/or at the level of the fluorogenic probe hybridization (*see, e.g.,* Fig. 4A, panel B). In both steps, the discrimination is based on the differential annealing of the perfectly matched, versus mismatched oligonucleotides. In the MethyLight technology, sequence discrimination at the PCR amplification level occurs by designing the primers and probe, or just primers, or just probes, to overlap potential sites of DNA methylation (*e.g.*, CpG dinucleotides). One approach is simply a fluorescence-based version of the MSP technique (Herman *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 9821-9826). Each oligonucleotide (primers and probe) can cover anywhere from zero to multiple CpG dinucleotides. Each CpG dinucleotide can result in two different sequence variations following bisulfite conversion, depending on whether that particular site was methylated (mCpG) or unmethylated (UpG). For example, if an oligonucleotide overlaps two CpG dinucleotides, then the number of possible sequence variants in the genomic DNA within the region covered by that oligonucleotide is 2 x 2 = 4. If both of the primers and the probe each overlap two CpGs, then the total number of variants contained within the sequence covered by the oligonucleotides is 4 x 4 x 4 = 64. In theory, one could design separate PCR reactions to analyze the relative amounts of each of these potential 64 sequence variants. However, significant methylation information can be derived from the analysis of a much smaller number of variants by designing reactions for the fully methylated and fully unmethylated molecules, which represent the two most extreme sequence variants this hypothetical example. The ratio between these two reactions or the ratio between the methylated reaction and a control reaction provides a measure of the prevalence of methylated molecules at this locus.

[0514] The MethyLight technology can also be modified to avoid sequence discrimination at the PCR amplification level. If the neither the primers nor the probe overlie any CpG dinucleotides, then the reaction represents unbiased amplification and can serve as a control for the amount of input DNA. One illustrative useful control reaction is one in which the entire amplicon is devoid of any CpG dinucleotides in the unconverted genomic sequence. When just the probe is designed to cover CpG dinucleotides, then sequence discrimination occurs solely at the level of probe hybridization. In this version, all sequence variants resulting from the sodium bisulfite conversion step are amplified with equal efficiency, as long as there is no amplification bias (*see, e.g.*, Wamecke *et al.* (1997) *Nucleic Acids Res.*, 25: 4422-1426). In this case, the design of separate probes for each of the different sequence variants associated with a particular methylation pattern (2 x 2 = 4 probes in the case of two CpGs) allows a quantitative determination of the relative prevalence of each sequence permutation in the mixed pool of PCR products.

[0515] In certain embodiments the analysis methods also provide PCR specific for unconverted DNA. This PCR may interrogate SNPs, mutations, and/or translocations, *etc.* In this regard, it is noted that the detection of mutations and methylation in a single cartridge is illustrated in Example 12 (*see, e.g.,* Figs. 27A and 27B). Detection of SNPs, mutations, translocations and the like can readily be accomplished by the inclusion of primers and probe sets specific for the detection of these targets.

Nested PCR and Multiplex PCR assays.

[0516] In certain embodiments methylated DNA can be detected using an PCR methods well known to those of skill in the art. In certain embodiments a nested PCR reaction is used to detect methylation targets. In one illustrative, but non-limiting, embodiment (*see, e.g.,* Example 4), a nested PCR protocol can be used where the first 15-20 cycle PCR reaction is not specific for methylation but only the converted DNA sequences (*i.e.,* they do not cross CpGs or in instances when they do a R= purine or Y= pyrimidine is used to catch both methylated and unmethylated template sequences). The second qPCR reaction (*e.g.*, a 45 cycle qPCR reaction) can contain both primers and probes that are specific for typically 2-3 methylated CpGs.

[0517] It will be noted that in certain embodiments, a MethyLight analysis is performed using a single probe (*see, e.g.,* Figure 4B). In this approach, using a single, *e.g.*, methylation-specific, probe (PR3) along with methylation specific forward (FW) and reverse (RV) primers, methylation specific PCR for the probe (PR3) provides a signal that is dependent on methylation and bisulfite conversion for the FW, RV and PR3 sequences.

[0518] In various embodiments, multiplexed PCR assays are contemplated. By way of illustration, Figure 4C illustrates a MethyLight approach using multiple probes (PR1, PR2, . . . PR5) that each target different regions. The combined signal from all the probes (PR1, PR2, PR3, PR4, and PR5) yields a measure of the amount/degree of methylation. In certain embodiments each probe has its own specific dye/fluor so that it is detectable independently of the other probes. Thus, even where one target is not methylated, a signal may still be detected, *e.g.*, if PR3 is not methylated there will be no/less signal from the remaining probes. Fig. 4D illustrates a MethyLight approach using multiple probes (PR1. . . PR5) that each target the same region, but provide signals for different methylation patterns. While the approach illustrated in Fig. 4C can provide detection from a larger region, this multi-probe approach on a single smaller region could be accomplished with sequence specific primers or probes interrogating the extent of methylation across a specific sequence after bisulfite conversion.

[0519] In certain embodiments a reverse complement multiplex assay for both strands can be used (*see, e.g.*, Fig. 26). Following bisulfite conversion, both strands lose their complementarity. Thus, primer and probe sets can be designed for one strand or the other, and result in unique amplicons. In addition to providing "more opportunities" for detection, this approach can potentially help with sensitivity (at LOD, if only one strand or the other ends up in the tube, this approach would ensure the signal gets picked up). This approach permits the multiplex assay to be expanded to detect different CpGs at the same promoter site. The reverse complement multiplex provides more opportunities to detect target methylation and to pick up heterogenous methylation.

[0520] The foregoing methods are illustrative and non-limiting. Using the teachings provided herein numerous variations of MSP and/or MethyLight analysis will be available to one of skill in the art and implementable on a reaction cartridge, *e.g.* as described herein.

Direct Sequencing

[0521] In certain embodiments methylation status of the DNA can be determined using direct sequencing methods. In certain embodiments, the method can utilize PCR and standard dideoxynucleotide DNA sequencing to directly determine the nucleotides resistant to bisulphite conversion (*see, e.g.*, Frommer *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89 (5): 1827-1831). In various embodiments primers are designed to be strand-specific as well as bisulphite-specific (*e.g.*, primers containing non-CpG cytosines such that they are not complementary to non-bisulphite-treated DNA), flanking (but not involving) the methylation site of interest. Therefore, it will amplify both methylated and unmethylated sequences, in contrast to methylation-specific PCR. All sites of unmethylated cytosines are displayed as thymines in the resulting amplified sequence of the sense strand, and as adenines in the amplified antisense strand. In certain embodiments nested PCR methods can be used to enhance the product for sequencing.

[0522] In certain embodiments the sequencing can be performed in the cartridge. In other embodiments, the cartridge can be coupled (*e.g.*, fluidic coupled) to a sequencing machine to provide the sequencing analysis. Alternatively, in certain embodiments, the amplified product can be manually transferred from the cartridge to the sequencing system.

High resolution melting analysis (HRM)

[0523] In certain embodiments high-resolution melting analysis (HRM) can be used to differentiate converted from unconverted bisulphite-treated DNA. HRM is a quantitative PCR technique in which the PCR amplicons are analyzed directly by temperature ramping and resulting liberation of an intercalating fluorescent dye during melting (*see, e.g.,* Wojdacz and Dobrovic (2007) *Nucleic Acids Res.* 35(6): e41). The degree of methylation, as represented by the C-to-T content in the amplicon, determines the rapidity of melting and consequent release of the dye. This method allows direct quantitation, but assesses methylation in the amplified region as a whole rather than at specific CpG sites.

Pyrosequencing

[0524] In certain embodiments pyrosequencing (sequencing by synthesis) can be used to analyze bisulphite-treated DNA without using methylation-specific PCR (*see, e.g.,* Colella *et al.* (2003). *BioTechniques* 35(1): 146-150; Tost *et al.* (2003) *BioTechniques* 35(1): 152-156; and the like). Sequencing by synthesis differs from Sanger sequencing in that it utilizes the detection of phosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. The DNA sequence is able to be determined by light emitted upon incorporation of the next complementary nucleotide by the fact that typically only one out of four of the possible A/T/C/G nucleotides are added and available at a time so that only one letter can be incorporated on the single stranded template (which is the sequence to be determined).

[0525] Following PCR amplification of the region of interest, pyrosequencing can be used to determine the bisulphite-converted sequence of specific regions (*e.g.*, CpG sites). In certain embodiments the ratio of C-to-T at individual sites can be determined quantitatively based on the amount of C and T incorporation during the sequence extension.

[0526] A modification of this technique can utilize allele-specific primers that incorporate single-nucleotide polymorphisms (SNPs) into the sequence of the sequencing primer(s) , thus allowing for separate analysis of maternal and paternal alleles (*see, e.g.,* Wong *et al.* (2006) *BioTechniques* 41(6): 734-739). This modification is particularly of use for genomic imprinting analysis.

Base-specific cleavage analysis.

[0527] In certain embodiments, base-specific cleavage/MALDI-TOF takes advantage of bisulphite-conversions by adding a base-specific cleavage step to enhance the information gained from the nucleotide changes (Ehrich *et al.* (2005) *Proc. Natl. Acad. Sci. USA*, 102 (44): 15785-15790). By first using *in vitro* transcription of the region of interest into RNA (by adding an RNA polymerase promoter site to the PCR primer in the initial amplification), RNase A can be used to cleave the RNA transcript at base-specific sites. RNase A cleaves RNA specifically at cytosine and uracil ribonucleotides and base-specificity is achieved by adding incorporating cleavage-resistant dTTP when cytosine-specific (C-specific) cleavage is desired, and incorporating dCTP when uracil-specific (U-specific) cleavage is desired. The cleaved fragments can then be analyzed by MALDI-TOF or other methods. Bisulphite treatment results in either introduction/removal of cleavage sites by C-to-U conversions or shift in fragment mass by G-to-A conversions in the amplified reverse strand. C-specific cleavage will cut specifically at all methylated CpG sites. By analyzing the sizes of the resulting fragments (*e.g.*, using MALDI-TOF, capillary electrophoresis, microchip electrophoresis, and the like), it is possible to determine the specific pattern of DNA methylation of CpG sites within the region, rather than determining the extent of methylation of the region as a whole.

Methylation-sensitive single-strand conformation analysis (MS-SSCA).

[0528] Methylation -sensitive single strand conformation analysis (MS-SSCA) is based on the single-strand conformation polymorphism analysis (SSCA) method developed for single-nucleotide polymorphism (SNP) analysis (Bianco *et al.* (1999) *Hum. Mutat.* 14(4): 289-293). SSCA differentiates between single-stranded DNA fragments of identical size but distinct sequence based on differential migration in non-denaturating electrophoresis. In MS-SSCA, this is used to distinguish between bisulphite-treated, PCR-amplified regions containing the CpG sites of interest. Although SSCA lacks sensitivity when only a single nucleotide difference is present, bisulphite treatment frequently makes a number of C-to-T conversions in most regions of interest, and the resulting sensitivity can be high. In certain embodiments MS-SSCA can also provide semi-quantitative analysis of the degree of DNA methylation based on the ratio of band intensities. Typically, however, MS-SSCA assesses all CpG sites as a whole in the region of interest rather than individual methylation sites.

Methylation targets.

[0529] As noted above, DNA methylation is of interest in a wide number of contexts. In certain embodiments, the amount of DNA methylation is of clinical interest particularly in oncology. Aberrant DNA methylation patterns (hypermethylation and hypomethylation compared to normal tissue) have been associated with a large number of human malignancies. Hypermethylation typically occurs at CpG islands in the promoter region and is associated with gene inactivation. A lower level of leukocyte DNA methylation is associated with many types of cancer (Zhang *et al.* (2011) Epigenetics, 6(3): 293-299). Global hypomethylation has also been implicated in the development and progression of cancer through different mechanisms. Typically, there is hypermethylation of tumor suppressor genes and hypomethylation of oncogenes (*see, e.g.,* Lund *et al.* (2004) *J. Biol. Chem.* 279(28): 29147-29154).

[0530] In this regard, it is noted that DNA methylation provides a prognostic indicator for Stage I Non–Small-Cell Lung Cancer (NSCLC). In particular, it was discovered that hypermethylation of five genes was significantly associated with shorter relapse-free survival (RFS) in stage I NSCLC: *HIST1H4F*, *PCDHGB6*, *NPBWR1*, *ALX1*, and *HOXA9*. A signature based on the number of hypermethylated events distinguished patients with high- and low-risk stage I NSCLC (*see, e.g.,* Sandoval *et al.* (2013) *J. Clin. Oncol.*, 4140-4147).

[0531] Similarly it has been observed that malignant gliomas may have the MGMT gene inactivated due to methylation of its promoter region. The prediction, born out by current research, is that by methylating the MGMT gene, a better response to chemotherapy can occur (as the tumor has no means to repair the DNA damage induced by the alkylating agent). In gliomas, MGMT promoter methylation is a favorable prognostic marker in the setting of either radiation or chemotherapy (*see, e.g.,* //neurosurgery.ucsd.edu/brain-tumor-research-mgmt/).

[0532] By way of further illustration, Table 3 illustrates various genes that are hypermethylated in certain cancers.

Table 3 shows illustrative, but non-limiting examples of genes hypermethylated in sporadic cancers (*see, e.g.,* Baylin (2005) *Nature Clinical Practice Oncology,* 2: S4-S11).

Gene or gene product	**Tumor type**
Rb	Retinoblastoma
APC	Colorectal and other cancers
p14/ARF	Colorectal cancer
p15/*CDKN2B*	Leukemias
p16/*CDKN2A*	Various cancers
BRCA1	Breast, ovarian cancer
VHL	Renal cell cancers

hMLH1	Colorectal, gastric, endometrial cancers
ER-α	Breast, colorectal, other cancers

[0533] In various illustrative, but non-limiting, embodiments measurement of methylation of any one of more of the promoters of the following genes is contemplated: *APC, ARF, CDKN2B, CDKN2A, BRCA1, VLH, hMLH1, MGMT. RASSF1A, ADAMTS1, BNC1, HIST1H3C, HOXB4, RASGRF2, TM6SF1*, *AKR1B1*, *HIST1H4F*, *PCDHGB6*, *NPBWR1*, *ALX1*, and *HOXA9*.

Pancreatic cancer.

[0534] In certain embodiments methylation status is determined for one or more promoters where methylation status is a marker for the presence and/or prognosis of pancreatic cancer. It was determined that the frequency of methylation of one or more of *ADAMTS1*, or *BNC1*, can be used to detect and/or stage pancreatic cancer. Thus, illustrative, but non-limiting methylation markers for pancreatic cancer include, but are not limited to *ADAMTS1* and/or *BNC1*. Illustrative primers and probes for the detection of methylation at the promoters of these genes are shown in Table 4, below (referencing Table 5 for particular sequences), and in Table 10 in Example 4). In certain embodiments primers and probes are provided for the detection of methylation in the forward strand of the converted DNA and/or for the detection of methylation in the reverse strand of the converted DNA.

Breast cancer.

[0535] In certain embodiments methylation status is determined for one or more promoters where methylation status is a marker for the presence and/or prognosis of breast cancer. Illustrative methylation markers for breast cancer include, but are not limited to *RASSF1A*, and/or *AKR1B1*, and/or *HOXB4*, and/or *HIST1H3C*, and/or *RASGRF2*, and/or *TM6SF1*. Illustrative primers and probes for the detection of methylation at the promoters of these genes are shown in Table 4, below (referencing Table 5 for particular sequences), and in Table 9 in Example 4.

[0536] In certain embodiments methylation status is determined for one or more promoters where methylation status is a marker for the presence or likelihood of lung cancer. Illustrative methylation markers for lung cancer include, but are not limited to *CDO1, SOX17, TAC1,* and/or *HOXA7*.

[0537] The methods described herein are not limited to determining methylation of the promoters of these genes. Using the methods described herein methylation of essentially any target of interest is possible.

[0538] It will be noted, however that measurement of DNA methylation need not be limited to measurement of methylation at CPG islands in promoters. For example, it has been demonstrated that gene body methylation can also alter gene expression and can provide a therapeutic target in cancer (*see, e.g.,* Yang *et al.* (2014) Cancer Cell, 26(4): 577-590).

[0539] Additionally, measurement of DNA methylation has prognostic/therapeutic applications for pathologies other than cancer. For example, aberrant methylation on regions on chromosomes 13, 18, 21, X, and Y can be used to diagnose Down syndrome (*see, e.g.,* Patsalis *et al.* (2012) *Exp. Opin. Biol. Ther.* 12(Suppl. 1): S155-S161). Because fetal DNA and maternal DNA are differentially methylated, cell-free DNA in maternal plasma can provide a source of fetal DNA, which can be obtained non-invasively and utilized to assess the methylation state of the aforementioned chromosomes (or other chromosomes or genes).

[0540] As noted above, in certain embodiments, the cartridges and methods described herein are also used to determine mRNA levels, *e.g.*, to determine expression of various methyltransferases. In certain embodiments, expression level of RNA is determined for a methyltransferase selected from the group consisting of *DNMT1, DNMT2, DNMT3A, DNMT3B*, and *TNMT3L*.

<u>Primers/Probes and multiplex analysis</u>

[0541] In various embodiments the methods described herein can involve nested PCR reactions and the cartridges described herein can contain reagents (*e.g.*, primers and probes) for such nested PCR reactions. For example, in certain embodiments, methylation is detected for one, two, three, four, five, or six genes (gene promoters). Since bisulfite conversion of a DNA changes cytosine resides to uracil, but leave 5-methyl cytosine residues unaffected, the forward and reverse strands of converted (bisulfite-converted) DNA are no longer complementary. Accordingly, it is possible to interrogate the forward and revers strands independently (*e.g.*, in a multiplex PCR reaction) to provide additional specificity and sensitivity to methylation detection. In such instances, assaying of a single target can involve a two-plex multiplex assay, while assaying of two, three, four, five, or six target genes can involve four-plex, six-plex, 8-plex, 10-plex, or 12-plex multiplex assays.

In certain embodiments the assays can be divided into two multiplex reactions, *e.g.*, to independently assay forward and reverse strands. However, it will be recognized that when split into multiple multiplex assays, the grouping of assays need not be by forward or reverse, but can simply include primer/probe sets that are most compatible for particular PCR reaction conditions.

[0542] As indicated above, numerous cancers can be identified, and/or staged and/or a prognosis therefor determined by the detection/characterization of the methylation state on the forward and/or reverse strand of gene promoters whose methylation (or lack thereof) is associated with a cancer. Illustrative gene (promoter) targets associated with various cancers are described above and shown below in Table 4. It will be recognized that methylation (forward strand and/or reverse strand) of one or more of the genes shown in Table 4 for each cancer can be determined to identify, and/or stage, and/or provide a prognosis for the indicated cancer. In certain embodiments methylation status of all of the genes shown for a particular cancer (forward and/or reverse strand) can be determined in a single multiplex PCR reaction.

Table 4. Illustrative primers and probes for the detection of methylation at the promoters of genes associated with various cancers using the devices and methods described herein. Primer and probe numbers refer to primer/probe numbers (primer/probe num) shown in Table 5, below.

Indication/Gene	External Primers	Internal Primers	Probe
Breast Cancer			
AKR1B1:	58 / 183	19 / 20	193
HIST1H3C:	42 / 43,	59 / 54	194
HOXB4:	186 / 187,	25 / 26	76
RASGRF2:	188 / 199,	192 / 14	67
RASSF1A:	189 / 1,	1 / 2	63
TM6SF1:	202 / 51,	31 / 57	77
BG:	175 / 158,	176 / 156	164
Pancreatic Cancer Set 1:			
BNC1	213 / 214,	221 / 222	229
ADAMTS1:	219 / 220,	227 / 228	265
ACTB:	102 / 103,	320 / 321	150
Pancreatic Cancer Set 2:			
BNC1:	217 / 218,	225 / 226	264
ADAMTS1:	215 / 216,	223 / 224	230
ACTB:	102 / 103,	320 / 321	150

Lung CPHD Set 2:			
CDO1:	283 / 284,	287 / 288	291
TAC1:	293 / 294,	386 / 388	301
SOX17:	303 / 304,	382 / 385	312
ACTB:	102 / 103,	320 / 321	150
Prostate:			
GSTP1:	233 / 234,	239 / 240	245
APC:	235 / 236,	241 / 242	246
PTGS2:	237 / 238,	243 / 244	247
ACTB:	102 / 103,	320 / 321	150
BRCA1:			
BRCA1:	328 / 329,	330 / 331	327
ACTB:	102 / 103,	320 / 321	150
MGMT:			
MGMT:	248b / 249b,	250 / 251	252
ACTB:	102 / 103,	320 / 321	150

[0543] Illustrative primers and probes for the detection of methylation at the promoters of various genes are shown below in Table 5, below, and in Tables 9 and 10 in Example 4. In certain embodiments these primers and/or probes are particularly suitable for use in a multiplex amplification.

Table 5. Illustrative primers and probes for the detection of methylation of various gene promoters. Note Y is C/T; R is A/G; C* is an optionally functionalized (*e.g.*, to alter probe T_m) C; T* is an optionally functionalized (*e.g.*, to alter probe T_m) T; A* is an optionally functionalized (*e.g.*, to alter probe T_m) A; N* is a nucleotide optionally a quencher; dP is a universal pyrimidine; dK is a universal purine.

Primer / Probe Num	Target	Type	Sequence	SEQ ID NO
1	RASSF1A	I	GCGTTGAAGTCGGGGTTC	2
2	RASSF1A	I	CCCGTACTTCGCTAACTTTAAACG	3
3	RASSF1A	P	ACAAACGCGAACCGAACGAAACCA-quencher/blocker	4
4	RASSF1A STD	I	fluor-TTAGGGTAGATTGTGGATATTAG	5
5	RASSF1A STD	I	ATACTAACAACTATCCAATACAAC	6
6	RASSF1A STD	P	fluor- (C*)AGGTTGAAATTAG(T-quencher)ATGTGTTATTTTGGTATGG	7
7	HIST1H3C	I	AATAGTTCGTAAGTTTATCGGCG	8
8	HIST1H3C	I	CTTCACGCCACCGATAACCGA	9

9	HIST1H3C	P	fluor-TACTTACGCGAAACTTTACCGCCGA-quencher/blocker	10
10	HIST1H3C STD	I	GATTTAGAGTTGGATGTGTGGAT	11
11	HIST1H3C STD	I	ACCACCATACTAATAATCAAATCTA	12
12	HIST1H3C STD	P	fluor-AAATATCACTCATCACCAAATAAATCCAA-quencher/blocker	13
13	RASGRF2	I	GTAAGAAGACGGTCGAGGCG	14
14	RASGRF2	I	ACAACTCTACTCGCCCTCGAA	15
15	RASGRF2	P	fluor-AACGAACCACTTCTCGTACCAACGA-quencher/blocker	16
16	RASGRF2 STD	I	TGTATGAGTTTGTGGTGAATAATG	17
17	RASGRF2 STD	I	AACTCACCATCAAACACTTTCCC	18
18	RASGRF2 STD	P	fluor-TACAAACCCAACATCCTCTATCTATTC-quencher/blocker	19
19	AKR1B1	I	GCGCGTTAATCGTAGGCGTTT	20
20	AKR1B1	I	CCCAATACGATACGACCTTAAC	21
21	AKR1B1	P	fluor-CGTACCTTTAAATAACCCGTAAAATCGA-quencher/blocker	22
22	AKR1B1 STD	I	TTTGTTGATGTTTTGTGGAAGTAAG	23
23	AKR1B1 STD	I	ATTCATCAATACTTTCAAATAACACA	24
24	AKR1B1 STD	P	fluor-(C*)AAATACATTATCC(T-quencher)ACCACTAACAATACA	25
25	HOXB4	I	CGGGATTTTGGGTTTTCGTCG	26
26	HOXB4	I	CGACGAATAACGACGCAAAAAC	27
27	HOXB4	P	fluor-AACCGAACGATAACGAAAACGACGAA-quencher/blocker	28
28	HOXB4 STD	I	GTTAGTTTTGTAGTGTATTGAGTAT	29
29	HOXB4 STD	I	CATCTTCCACAATAAACTTCCAATT	30
30	HOXB4 STD	P	fluor-TAACTCCACCTATTCTACCTACCATTT-quencher/blocker	31
31	TM6SF1	I	CGTTTAGCGGGATGCGGTGA	32
32	TM6SF1	I	ACACGAAAACCCCGATAACCG	33
33	TM6SF1	P	fluor-AAACACTCATCGCAACCGCCGCG-quencher/blocker	34
34	TM6SF1 STD	I	TTAGATGTTGATTGGTTGTGTTTG	35
35	TM6SF1 STD	I	ATCATCATAAAACTCAACAATCAATT	36
36	TM6SF1 STD	P	fluor-CCAAACATCAAATCTTTAACTTTTACCAA-quencher/blocker	37
37	RASSF1A STD	P	fluor-AGGTTGAAATTAGTATG(T-quencher)GTTATTTTGGTATGG-quencher/blocker	38

38	RASSF1A STD	P	fluor-AGGTTGAAATTAGTATGTGT(T-quencher)ATTTTGGTATGG-quencher/blocker	39
39	RASSF1A STD	P	fluor-AGGTTGAAATTAGTATGTGTTA(T-quencher)TTTGGTATGG-quencher/blocker	40
40	RASSF1A	E	GTTTTATAGTTTTTGTATTTAGG	41
41	RASSF1A	E	AACTCAATAAACTCAAACTCCC	42
42	HIST1H3C	E	GTGTGTGTTTTTATTGTAAATGG	43
43	HIST1H3C	E	ATAAAATTTCTTCACRCCACC	44
44	RASGRF2	E	GAGGGAGTTAGTTGGGTTAT	45
45	RASGRF2	E	CCTCCAAAAAATACATACCC	46
46	AKR1B1	E	GTGTAATTAATTAGAAGGTTTTTT	47
47	AKR1B1	E	AACACCTACCTTCCAAATAC	48
48	HOXB4	E	TTAGAGGYGAGAGAGTAGTT	49
49	HOXB4	E	AAACTACTACTAACCRCCTC	50
50	TM6SF1	E	AGGAGATATYGTTGAGGGGA	51
51	TM6SF1	E	TCACTCATACTAAACCRCCAA	52
52	RASSF1A STD	I	TTAGGGTAGATTGTGGATATTAGATAGG	53
53	RASSF1A STD	I	TAATACTAACAACTATCCAATACAACAC	54
54	HIST1H3C	I	CCGATAACCGAAACGCTCTTAC	55
55	AKR1B1	I	GCGTTAATCGTAGGCGTTT	56
56	TM6SF1	I	GTTTAGCGGGATGCGGTG	57
57	TM6SF1	I	ACACGAAAACCCCGATAAC	58
58	AKR1B1	E	GYGTAATTAAT(T*)AGAAGGTTTTTT	59
59	HIST1H3C	I	TCGTACGAAGTAAATAGTTCGTAAG	60
60	HIST1H3C	E	GGATTTTTGAAATATTATAGGATTAATTAG	61
61	RASSF1A	E	GTTTTATAGTT(T*)TTGTATTTAGG	62
62	RASSF1A	P	fluor-ACAAACGCGA(N*)ACCGAA(C**)GAAACCA-quencher/blocker	63
63	RASSF1A	P	fluor- (C*)TGGTTTCGT(T-quencher)CGGT(T*)CGCG-quencher/blocker	64
64	RASSF1A STD	P	fluor- (C*)AGGTTGAAATTAGTA(T-quencher)GTGTTAT(T*)TTGG(T*)ATGG-quencher/blocker	65
65	HIST1H3C	P	fluor-CAAACTACTTACGCGAAACTT(T*)ACCGCC-quencher/blocker	66
66	HIST1H3C STD	P	fluor-AAATATCACTCA(T*)CACCAAA(N*)TAAA(T*)CCAA-quencher/blocker	67
67	RASGRF2	P	fluor-AAACGAACCACTTCTCG(T*)ACCAACGAC-quencher/blocker	68
68	RASGRF2 STD	P	fluor-CAAACCCAACATCCTC(T*)ATC(T*)ATTC-quencher/blocker	69
69	AKR1B1	P	fluor-	70

			A(C*)GCGTACCTTT(N*)AAA(T*)AACCCG (T*)AAAATCG-quencher/blocker	
70	AKR1B1	P	fluor-A(C*)GCGTACCTT(T-quencher)AAA(T*)AACCCG(T*)AAAATCG-quencher/blocker	71
71	AKR1B1 STD	P	fluor- (C*)AAATACATTATCC(T-quencher)ACCAC(T*)AACAA(T*)ACA-quencher/blocker	72
72	HOXB4	P	fluor-AACCGAACGATAACGAAAA(C**)GACGA-quencher/blocker	73
73	HOXB4 STD	P	fluor-TAACTCCACCTATTC(T*)ACCT(N*)ACCA(T*)TT-quencher/blocker	74
74	TM6SF1 STD	P	fluor-CAAACATCAAATCT(T*)TAAC(T*)TT(T*)AC-quencher/blocker	75
75	AKR1B1	P	fluor- (C*)A(C*)GCGTACCT(T-quencher)TAAA(T*)AACCCG(T*)AAAATCG-quencher/blocker	76
76	HOXB4	P	fluor-AACCGAACGA(T*)AACGAAA(N*)ACGACGAA-quencher/blocker	77
77	TM6SF1	P	fluor-AAACACTCATCGCAACCGCCGCG-quencher/blocker	78
78	ACTB	P	fluor-TAACCACCACCCAACACA(C**)AATAAC-quencher/blocker	79
79	ALU Long Set 1	P	fluor-CCCAACTACT(T*)AAAAAAC(T*)AAAAC-quencher/blocker	80
80	ALU Short Set 1	P	fluor-CACCTAAAA(T*)CAAAAATT(T*)AAAACC-quencher/blocker	81
81	ALU Long Set 2	P	fluor-CAAATAATTCTCC(T*)ACCTCAACC(T*)C-quencher/blocker	82
82	ALU Short Set 2	P	fluor-CTTAACTCAC(T*)ACAACCTC(T*)ACC-quencher/blocker	83
83	INSL6	P	fluor-CAAACCGAACGACGCGCACAAACAC-quencher/blocker	84
84	ACTB	E	GTATATAGGTTGGGGAAGTTTG	85
85	ACTB	E	AACTATACTCAACCAATAAAACC	86
86	ALU Long Set 1	E	TGTTATTTAGGTTGGAGTGTAG	87
87	ALU Long Set 1	E	TAATAACTCATACCTATAATCCC	88
88	ALU Long Set 1	I	GGTTGGAGTGTAGTGGTATAATTTTAG	89
89	ALU Long Set 1	I	TAATAACTCATACCTATAATCCCAACAC	90
90	ALU Short Set 1	E	GTAGAGATAGGGTTTTATTATGTTG	91
91	ALU Short Set 1	I	GGTTTTATTATGTTGGTTAGGTTGG	92
92	ALU Long Set 2	E	GTATTTTGGGAGGTTAAGGTAG	93

93	ALU Long Set 2	E	ATCTTACTCTTATTACCCAAAC	94
94	ALU Long Set 2	I	GGTTAAGGTAGGTAGATTATTTGAGG	95
95	ALU Long Set 2	I	ATCTTACTCTTATTACCCAAACTAAAATAC	96
96	ALU Short Set 2	E	GTTATTTAGGAGGTTGAGGTAG	97
97	ALU Short Set 2	E	GAGGTAGGAGAATTATTTGAATTTAGG	98
98	INSL6	E	ATTTGAGATTTTTGAGTTGG	99
99	INSL6	E	AACCCTACTCCCTATCTACG	100
100	INSL6	I	GCGCGCGTTTTTTTTGAAG	101
101	INSL6	I	GGCGTAGATAGGGAGTAGGGTT	102
102	ACTB	I	GTGATGGAGGAGGTTTAGTAAGTT	103
103	ACTB	I	CCAATAAAACCTACTCCTCCCTTAA	104
104	RASSF1A STD	P	fluor- (C*)(C*)ATACCAAAA(T-quencher)AACACA(T*)CTAAT(T*)TCAACCT-quencher/blocker	105
105	AKR1B1 STD	P	fluor- (C*)AAATACAT(T*)ATCC(T-quencher)ACCAC(T*)AACAA(T*)ACA-quencher/blocker	106
106	AKR1B1	P	fluor- (C*)ACGCGTACCTT(T-quencher)AAA(T*)AACCCG(T*)AAAATCG-quencher/blocker	107
107	AKR1B1	P	fluor- (C*)ACGCGTACCTT(T*)AAA(T-quencher)AACCCG(T*)AAAATCG-quencher/blocker	108
108	RASSF1A UM	P	fluor-CTAACAAACA(C-quencher)AAA(C**)CAAA(C**)AAAACCA-quencher/blocker	109
109	RASSF1A UM	P	fluor-CTAACAAACA(C**)AAA(C-quencher)CAAA(C**)AAAACCA-quencher/blocker	110
110	HIST1H3C UM	P	fluor-AACTACTTACA(C**)AAAACTT(N*)TAC(C**)ACCAA-quencher/blocker	111
111	HIST1H3C UM	P	fluor-AACTACTTA(C**)ACAAAA(C**)TTTACCAC-quencher/blocker	112
112	RASGRF2 UM	P	fluor-AAACAAACCAC(T*)TCTCA(T*)ACCAACAAC-quencher/blocker	113
113	AKR1B1 UM	P	fluor- (C*)ACATACCTTTAAA(T-quencher)AACCCA(T*)AAAA(T*)CAAC-quencher/blocker	114
114	AKR1B1 UM	P	fluor- (C*)ACATACCTT(T-quencher)AAA(T*)AACCCA(T*)AAAATCAAC-quencher/blocker	115
115	HOXB4 UM	P	fluor-CAACAAAAACCCAAAA(T*)CCCAAC(N*)AAACCACA-quencher/blocker	116
116	HOXB4 UM	P	fluor-CAAAATCCCAA(C**)AAACCA(C**)ATAACA-quencher/blocker	117

117	TM6SF1 UM	P	fluor-AAACACTCATCACAACCA(C**)CACACC-quencher/blocker	118
118	AKR1B1 UM	I	TGGTGTGTTAATTGTAGGTGTTTT	119
119	AKR1B1 UM	I	CCCAATACAATACAACCTTAACC	120
120	HOXB4 UM	I	GTGGTGTGTATTGTGTAGTGTTA	121
121	HOXB4 UM	I	CAAACCAAACAATAACAAAAACAAC	122
122	TM6SF1 UM	I	TGTTTAGTGGGATGTGGTGAAG	123
123	TM6SF1 UM	I	ACACAAAAACCCCAATAACCACA	124
124	RASSF1A UM	I	GTTTAAAGTTAGTGAAGTATGGGTTT	125
125	HIST1H3C UM	I	TGTATGAAGTAAATAGTTTGTAAGTTTATTGG	126
126	AKR1B1 STD	I	TTTGTTGATGTTTTGTGGAAG(T*)AAG	127
127	AKR1B1 STD	I	ATTCATCAATACTTTCAAA(T*)AACACA	128
128	RASGRF2	P	fluor-AAACGAACCACTTCTCG(T*)ACCAACGAC-quencher/blocker	129
129	RASGRF2 STD	P	fluor-CAAACCCAACATCCTC(T*)ATC(T*)ATTC-quencher/blocker	130
130	TM6SF1	P	fluor-AAACACTCATCGCAACCGCCGCG-quencher/blocker	131
131	TM6SF1 STD	P	fluor-CCAAACATCAAATCT(T*)TAACTT(T*)TACCAA-quencher/blocker	132
132	TM6SF1	P	fluor-AAACACTCATCGCAACCGCCGCG-quencher/blocker	133
133	RASSF1A UM	I	GGTGTTGAAGTTGGGGTTTG	134
134	RASSF1A UM	I	CCCATACTTCACTAACTTTAAAC	135
135	HIST1H3C UM	I	GTAAATAGTTTGTAAGTTTATTGGTG	136
136	HIST1H3C UM	I	TTTCTTCACACCACCAATAACCAA	137
137	RASGRF2 UM	I	GAGTAAGAAGATGGTTGAGGTG	138
138	RASGRF2 UM	I	CAACAACTCTACTCACCCTCAA	139
139		P	fluor-TCCCAACTACT(T*)AAAAAAC(T*)AAAAC-quencher/blocker	140
140	ALU Long Set 1	P	fluor-TCCCAACTACT(T*)AAAAAAC(T*)AAAAC-quencher/blocker	141
141	ALU Long Set 1	P	fluor-TCCCAACTACT(T*)AAAAAAC(T*)AAAAC-quencher/blocker	142
142	ALU Long Set 1	P	fluor-TCCCAACTACT(T*)AAAAAAC(T*)AAAAC-quencher/blocker	143
143	ALU Long Set 1	P	fluor-TCCCAACTACT(T*)AAAAAAC(T*)AAAAC-quencher/blocker	144
144	HMBS	I	GGATAAGATTTTTGATATTGTATTTTTAAGG	145

145	HMBS	I	CATATTCAAACTCCTTAATAAACAAACTTTTCTC	146
146	HMBS	P	fluor-CCGAACAAAAAAAA(C-quencher)CTAAA(T*)AAATCCC(T*)TC-quencher/blocker	147
147	ACTB	P	fluor-CCACCACCCAACACACAA(T*)AACAAACAC-quencher/blocker	148
148	ACTB	I	GGTTTAGTAAGTTTTTTGGATTGTG	149
149	ACTB		CCTTAAAAATTACAAAAACCACAAC	150
150	ACTB	P	fluor-CCACCACCCAACA(C-quencher)ACAA(T*)AACAAACAC-quencher/blocker	151
151	ACTB	P	fluor-CCACCACCCAAC(N*)ACA(C**)AATAA(C**)AAACAC-quencher/blocker	152
152	ACTB	P	fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-quencher/blocker	153
153	BG	I	TTCAGTGCCGGTTGGTAATGTAA-quencher/blocker	154
154	BG	I	CAACAACTTTAATACCTGTTTCAAGGA	155
155	BG conv	I	GGTATTTTTGTATTTGTTGGTGTTG	156
156	BG conv	I	CATACATACACCAAACAATTCATTC	157
157	BG conv	E	GTATGGTGGTATTTTTGTATTTGTTG	158
158	BG conv	E	CACACATACATACACCAAACAATTC	159
159	BG	P	fluor-AAGATCCGATTCACAGA(N*)CAAGCTCCGTCA-quencher/blocker	160
160	BG	I	fluor-AAGATCCGATTCACAGA(N*)CAAGCTCCGTCA-quencher/blocker	161
161	BG conv	P	fluor- (C*)AAAATCATTT(C-quencher)CTT(C**)ACAAATA(C**)ACTC-quencher/blocker	162
162	BG conv	P	fluor-CCAAATACCA(T-quencher)AACCA(T*)TTTATTAA(T*)AACAC-quencher/blocker	163
163	BG conv	P	fluor-AAAATCATTTCCTT(C**)ACA(N*)AATA(C**)ACTC-quencher/blocker	164
164	BG conv	P	fluor-CCAAATACCA(T*)AACCAT(N*)TTTATTAA(T*)AACAC-quencher/blocker	165
165	short HMBS	I	CCCTAGTATGCTAGGTCTCTTGCTGGGA	166
166	short HMBS	I	CAGCCTCTCTGAGGGTTTAAGCCCA	167
167	short HMBS	P	fluor-TCAGCC(T*)ATC(T*)GACACCCCGGG-quencher/blocker	168
168	short β-Globin	I	GACTCCTGAGGAGAAGTCTGCCGTTA	169
169	short β-Globin	I	CCTTGATACCAACCTGCCCAGGG	170

170	short β-Globin	P	fluor-AGGTGAACG(T*)GGATGAAGT(T*)GGTGGTG-quencher/blocker	171
171	short BG	I	CAACATCGCGCAAGAGCACGG	172
172	short BG	I	CGTTTCCTTCACGAGTACGCTCTCCGA	173
173	short BG	P	fluor-ACCGGCGAA(T*)ACAGAGA(T*)ACCG-quencher/blocker	174
174	ACTB	P	fluor-CC(A*)CC(A*)CCCAAC(N*)ACA(C**)AATAA(C**)AAACAC-quencher/blocker	175
175	BG conv	I	GTTGGTGTTGGAGAGTGTATTTG	176
176	BG conv	I	GGAGAGTGTATTTGTGAAGGAAATG	177
177	BG conv	I	GGAAATGATTTTTTTATGAGATGAGTG	178
178	ACTB	P	fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-quencher/blocker	179
179	ACTB	P	fluor-CCACCACCCAACACA(N*)CAA(T*)AACAAACAC-quencher/blocker	180
180	ACTB	P	fluor-CCACCACCCAACACACAA(T*)AACAAACAC-quencher/blocker	181
181	ACTB	I	GATGGAGGAGGTTTAGTAAGTTTTT	182
182	ACTB	I	AATAAAACCTACTCCTCCCTTAAAAA	183
183a	AKR1B1	E	CTTACCATAACTACTAC(dK)CTCC	184
183b	AKR1B1	E	CTTACCATAACTACTACRCTCC	185
184	HIST1H3C	E	GTGTGTGTTTTTATTGTAAATGGT	186
185a	HIST1H3C	E	AAC(dK)ATAAC(dK)ATAAAATTTCTTCAC	187
185b	HIST1H3C	E	AACRATAACRATAAAATTTCTTCAC	188
186a	HOXB4	E	GTTTGT(dP)GGGATTTTGGGT	189
186b	HOXB4	E	GTTTGTYGGGATTTTGGGT	190
187a	HOXB4	E	CC(dK)AACTCC(dK)AAAAAAAAACC	191
187b	HOXB4	E	CCRAACTCCRAAAAAAAAACC	192
188a	RASGRF2	E	GGTATTAAG(dP)G(dP)GGTTTTTTG	193
188b	RASGRF2	E	GGTATTAAGYGYGGTTTTTTG	194
189a	RASSF1A	E	GT(dP)GTTTAGTTTGGATTTTGG	195
189b	RASSF1A	E	GTYGTTTAGTTTGGATTTTGG	196
190	TM6SF1	E	TTTCGAAGGGTAAGCGTTAAG	197
191a	TM6SF1	E	AACATAAATAACC(dK)AAA(T*)AACC	198
191b	TM6SF1	E	AACATAAATAACCRAAA(T*)AACC	199
192	RASGRF2	I	CGGTTTTTTGAGTAAGAAGACGGTC	200
193a	AKR1B1	P	fluor-TACCTTTAAA(T-quencher)AACCC(dK)(T*)AAAA(T*)CGACAA-quencher/blocker	201
193b	AKR1B1	P	fluor-TACCTTTAAA(T-quencher)AACCCR(T*)AAAA(T*)CGACAA-	202

			quencher/blocker	
194a	HIST1H3C	P	fluor-ATAACAAACTACT(T*)AC(dK)CGAAAC(T*)TTAC-quencher/blocker	203
194b	HIST1H3C	P	fluor-ATAACAAACTACT(T*)ACRCGAAAC(T*)TTAC-quencher/blocker	204
195a	HOXB4	P	fluor-AACAAACC(dK)AA(C**)GA(T*)AAC(N*)AAAAC-quencher/blocker	205
195b	HOXB4	P	fluor-AACAAACCRAA(C**)GA(T*)AAC(N*)AAAAC-quencher/blocker	206
196	RASGRF2	P	fluor-CACATTCTAA(T*)AAAAAAC(N*)AACCAC(T*)TC-quencher/blocker	207
197a	RASSF1A	P	fluor-AACC(dK)AA(C**)GAAA(C-quencher)CA(C**)AAAAC-quencher/blocker	208
197b	RASSF1A	P	fluor-AACCRAA(C**)GAAA(C-quencher)CA(C**)AAAAC-quencher/blocker	209
198	TM6SF1	P	fluor-CAAAAACAC(T*)CATC(N*)CAACCGCC-quencher/blocker	210
199	RASGRF2	E	ACAACCCTCCAAAAAATACATA	211
200	BG conv	P	fluor-CCAAATACCATAACCA(T*)TTTATTAA(T*)AACAC-quencher/blocker	212
201	BG conv	P	fluor-CCAAATACCATAACCA(T*)TTTATTAA(T*)AACAC-quencher/blocker	213
202a	TM6SF1	E	TTT(dP)GAAGGGTAAG(dP)GTTAAG	214
202b	TM6SF1	E	TTTYGAAGGGTAAGYGTTAAG	215
203a	TM6SF1	E	CAACAC(dK)AAAACCCC(dK)ATA	216
203b	TM6SF1	E	CAACACRAAAACCCCRATA	217
204	KRAS Multi	E	CCTGCTGAAAATGACTGAATATAACCGCTAAGAACCTCTCGGTCAGCTGAT	218
205	KRAS Multi	E	CCTGCTGAAAATGACTGAATATAAAGTCTCATTATAATCGTTCGAGCTGTT	219
206	KRAS Multi	E	CCTGCTGAAAATGACTGAATATAAGCAGACTTGGCGGTAGGTCCGAGCTTG	220
207	KRAS Multi	E	CCTGCTGAAAATGACTGAATATAAGTATCCTGAGCACGGTTGCGAGCTGCT	221
208	KRAS Multi	I	CTCTTGCCTACGCC(N*)CCGCTAAGAACCTCTCGGTC	222
209	KRAS Multi	I	CTCTTGCCTACGCC(N*)AGTCTCATTATAATCGTTCG	223
210	KRAS Multi	I	CTCTTGCCTACGCC(N*)GCAGACTTGGCGGTAGGTCC	224
211	KRAS Multi	I	CTCTTGCCTACGCC(N*)GTATCCTGAGCACGGTTGCG	225
212	ACTB	P	fluor-CCACCACCCAACACACAA(T*)AACAAAC	226

			AC-quencher/blocker	
213	BNC1	E	CCCRCAAACCRCGAAAACCTC	227
214	BNC1	E	GTTTTTTTTYGGGAGAGGTAAATA	228
215	ADAMTS1	E	CRCCTCCRAAACTAAAACAAC	229
216	ADAMTS1	E	GGGTTATTGTAAAGTTAGGGTG	230
217	BNC1	E	GAGGT(dP)GTGGTTTT(dP)GTAGAT	231
218	BNC1	E	AAAC(dK)CCAAAAAACTTCAAAAC	232
219	ADAMTS1	E	TTTTGTTGGGATAAGAAG(dP)GTTT	233
220	ADAMTS1	E	ACCAAAAACTATTACAAAACCAAA	234
221	BNC1	I	CCGACGACCGACG	235
222	BNC1	I	GGGAGAGGTAAATATCGATAC	236
223	ADAMTS1	I	CGCGAAAATTAATACCTAACG	237
224	ADAMTS1	I	TTAGGGTGCGTTATCGGAC	238
225	BNC1	I	CGGAGGTGTTTGTTTTCGTC	239
226	BNC1	I	CGAAAAAAACAAACACCGACACG	240
227	ADAMTS1	I	CGTTTTCGGGGTTGAGGTAAC	241
228	ADAMTS1	I	CCAAAATACGCTACCGAACGA	242
229	BNC1	P	fluor- AAAAT(A*)TCT(A*)(C**)CCC(C**)(dK)CC- quencher/blocker	243
230	ADAMTS1	P	fluor- TATTACTCACTCTAC(T*)CAAAAC(T*)CT CC-quencher/blocker	244
231	BNC1	P	fluor- ATATCTTTTACCAACAAA(T*)ACCT(T*)C AAA-quencher/blocker	245
232	ADAMTS1	P	fluor- GTTTT(dP)GTTTTGGTTGCGA(T*)GTTGT- quencher/blocker	246
233	GSTP1	E	GGGATTTTTTAGAAGAG(dP)GGT	247
234	GSTP1	E	TACTCACTAATAAC(dK)AAAAC(T*)AC	248
235	APC	E	GGTTTTGTGTTTTATTG(dP)GGAG	249
236	APC	E	CCTAAC(dK)AACTACACCAATACAA	250
237	PTGS2	E	GAGAGGGGATTTTTTG(dP)GTTT	251
238	PTGS2	E	CC(dK)AAAACCAATTCTAAACTAATC	252
239	GSTP1	I	TTTTTAGAAGAGCGGTCGGC	253
240	GSTP1	I	CTAATAACGAAAACTACGACGACG	254
241	APC	I	TTGTGTTTTATTGCGGAGTGC	255
242	APC	I	AACCACATATCGATCACGTACG	256
243	PTGS2	I	GCGTTTTCGGATTTTAGGGTC	257
244	PTGS2	I	AACTAATCGCCTTAAATAAAATACCG	258
245	GSTP1	P	fluor- CCTCC(dK)AACCTTA(T*)AA(N*)AAA(T*) AATCCC-quencher/blocker	259
246	APC	P	fluor-	260

			AAAAAC(dK)CCCTAATCC(N*)CA(T*)CCAAC-quencher/blocker	
247	PTGS2	P	fluor-CACTTAACTTCCTC(T*)CCAAAAATC(T*)AAAC-quencher/blocker	261
248a	MGMT	E	GTTTT(T*)AGAA(dP)G(T*)TTTG(dP)GTTT	262
248b	MGMT	E	GTTTT(T*)AGAAYG(T*)TTTGYGTTT	263
249a	MGMT	E	AAAAAAC(T*)CC(dK)CACTCTTCC	264
249b	MGMT	E	AAAAAAC(T*)CCRCACTCTTCC	265
250	MGMT	I	TTTCGACGTTCGTAGGTTTTCGC	266
251	MGMT	I	GCACTCTTCCGAAAACGAAACG	267
252a	MGMT	P	fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-quencher/blocker	268
252b	MGMT	P	fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-quencher/blocker	269
264	BNC1	P	fluor-ATATCTTTTACCAA(C-quencher)AAA(T*)ACCT(T*)CAAA-quencher/blocker	270
265	ADAMTS1	P	fluor-GTTTT(dP)GTTTTGGTTGCGA(T*)GTTGT-quencher/blocker	271
283	CDO1	E	GGAGATAA(dP)GGGGTTTTTGG	272
284	CDO1	E	CACTAAAAATATACCAAC(dK)ACC	273
285	CDO1	E	GGAGAGTTATTTAAGAAAGGTGG	274
286	CDO1	E	AAAATTAC(dK)C(dK)AAACCCAC	275
287	CDO1	I	CGTGTTCGTAGGGTTTTTTCGTTTTC	276
288	CDO1	I	CCAACGACCCTCGAAAAAAAAACG	277
289	CDO1	I	GATTTTGCGGGTACGGTTTACGC	278
290	CDO1	I	GATCCCTAAAACGCCGAAAACAACG	279
291	CDO1	P	fluor- (C*)GTTATTTTT(T-quencher)TTGGG(T*)GGTT(T*)TTCG-quencher/blocker	280
292	CDO1	P	fluor-C(dK)AAAAACCACC(C-quencher)AAAAAAAA(T*)AAC-quencher/blocker	281
293	TAC1	E	GGATAAATAT(dP)GTAAGGTATTGAG	282
294	TAC1	E	CGAAATACTAAATTTCTCTAATTCCTC	283
295	TAC1	E	GAGTTTTTTTGGTTTTTT(dP)GAG	284
296	TAC1	E	CTAAAATAAATACC(dK)CAAAACAC	285
297	TAC1	I	CGCGTTCGGATTTTTTTTCGGC	286
298	TAC1	I	AAATTTCTCTAATTCCTCCGAACGCACG	287
299	TAC1	I	GCGTACGTTGGTCGTTTCGTATTTTC	288
300	TAC1	I	GCAAAACACTAAACAAACGAAAAAACGCG	289
301	TAC1	P	fluor-GTAGTTAT(dP)GAGAG(T*)G(N*)GGAGCG	290

			A(T*)TAG-quencher/blocker	
302	TAC1	P	fluor-CTAATC(dK)CTCCGCAC(T*)CTC(N*)A(T*)AACTAC-quencher/blocker	291
303	SOX17	E	GTTTGGAG(dP)GTTATGAGTAG	292
304	SOX17	E	CTTCATATCCCC(dK)ATAAAACTC	293
305	SOX17	E	GGGTTTTTAGTCGGTTTAGTG	294
306	SOX17	E	CTAAAAC(dK)TAAAACTC(dK)AACC	295
307	SOX17	I	GATTTAGAGCGCGTTGTTCGC	296
308	SOX17	I	CATATCCCCGATAAAACTCAACGACTCG	297
309	SOX17	I	GTCGGTTTAGTGATATTGCGGGC	298
310	SOX17	I	CCACGACCTAAACGTAAACCTAACG	299
311	SOX17	P	fluor-GATGGT(dP)GGGTTGGGTT(T*)TTGTTTTTGG-quencher/blocker	300
312	SOX17	P	fluor-CCAAAAACAAAAACCCAA(C**)CCGACCATC-quencher/blocker	301
313	CDO1	P	fluor-(C*)GTATATTTT(dP)GGTT(T*)TTT(N*)GGGT(T*)TCG-quencher/blocker	302
314	CDO1	P	fluor-C(dK)AAACC(C**)GAAAAAA(C**)C(N*)AAAATATAC-quencher/blocker	303
315	TAC1	P	fluor-GGTAGTTGT(dP)G(T*)CGGGAAGGAGGTTCG-quencher/blocker	304
316	TAC1	P	fluor-C(dK)AACCTCCTTCCCGAC(N*)ACAAC(T*)ACC-quencher/blocker	305
317	SOX17	P	fluor-GGTTTTTTTTGTA(T*)AGATGTGGT(T*)AATGG-quencher/blocker	306
318	SOX17	P	fluor-CCATTAACCACA(T*)CTA(T*)ACAAAAAAAACC-quencher/blocker	307
319	SOX17	E	GGTTTGGTTTATAG(dP)GTATTTAGG	308
320	ACTB	I	GAGGTTTAG(T*)AAGTTTTTTGGATTGTG	309
321	ACTB	I	CCCTTAAAAAT(T*)ACAAAAACCACAAC	310
322	BRCA1	E	GTAGATTGGGTGGTTAATTTAGAG	311
323	BRCA1	E	CTATAATTCCC(dK)C(dK)CTTTTC	312
324	BRCA1	I	GGTGGTTAATTTAGAGTTTCGAGAGAC	313
325	BRCA1	I	CGTTACCACGAAAACCAAAAAACTACCG	314
326	BRCA1	P	fluor-GATTTCGTATTT(T*)GAGAGG(T*)TGTTGTTTAG-quencher/blocker	315
327	BRCA1	P	fluor-CTAAACAACAACC(T*)CTCAAAA(T*)ACGAAATC-quencher/blocker	316

328	BRCA1	E	GGTAGATTGGGTGGTTAATTTAGAG	317
329	BRCA1	E	CCAAAAAATCTCAACRAACTC	318
330	BRCA1	I	GGGTGGTTAATTTAGAGTTTCGAGAGAC	319
331	BRCA1	I	ACCACGAAAACCAAAAAACTACCG	320
336	MGMT	E	GGGATTTTGTTTAAGTATGTTAAAGG	321
337	MGMT	E	CCTACCTTACCTCTAAATACCAACC	322
338	MGMT	I	GTATGTTAAAGGGTTGTTGTAAGTTAAG G	323
339	MGMT	I	CCTCTAAATACCAACCCCAAACC	324
340	MGMT	P	fluor-CCAACTACTC(C-quencher)AAAAAACTTCCAAAAACC-quencher/blocker	325
341	MGMT	P	fluor-CCAAC(T*)ACTC(C-quencher)AAAAAAC(T*)TCCAAAAACC-quencher/blocker	326
342	MGMT	I	GTATGTTAAAGGGTTGT(T*)GTAAGTTAA GG	327
343	MGMT	I	CCTCTAAATACCAA(C**)CCCAAACC	328
380	ACTB	P	fluor-CCACCACCCAACACACAA(T*)AACAAAC AC-quencher/blocker	329
381	ACTB	P	fluor-CCACCACCCAACACACAA(T*)AACAAAC AC-quencher/blocker	330
382	SOX17	I	ATTTAGAGCGCGTTGTTCGC	331
383	SOX17	I	ATATCCCCGATAAAACTCAACGACTCG	332
384	SOX17	I	TATCCCCGATAAAACTCAACGACTCG	333
385	SOX17	I	ATCCCCGATAAAACTCAACGACTCG	334
386	TAC1	I	GCGTTCGGATTTTTTTTTCGGC	335
387	TAC1	I	TTTCTCTAATTCCTCCGAACGCACG	336
388	TAC1	I	CTCTAATTCCTCCGAACGCACG	337
389	SOX17	I	GTGACGATTAGAGTTAGATTTAGAGCGC	338
390	TAC1	P	fluor-GTAGTTATCGAGAG(T*)GCGGAGCGA(T*)TAG-quencher/blocker	339
391	SOX17	P	fluor-CCAACCCGACCATCACCGCGAACAAC-quencher/blocker	340
392	BG converted	I	GGAGAGTGTATTTG(T*)GAAGGAAATG	341
393	BG converted	I	CATACATACACCAAACAA(T*)TCATTC	342
394	BG converted	P	fluor-CCAAATACCA(T*)AACCATTTTATTAA(T*)AACAC-quencher/blocker	343
395	BG converted	P	fluor-CCAAATACCA(T*)AACCATTTTATTAA(T*)AACAC-quencher/blocker	344
396	GSTP1 (Fwd)	E	GGTTTYGTTGGGGATTTG	345
397	GSTP1 (Fwd)	E	ACCRCTCTTCTAAAAAATCC	346

398	GSTP1 (Fwd)	I	AGGTTTTTTCGGTTAGTTGCGC	347
399	GSTP1 (Fwd)	I	AACGTCGACCGCAAAAAAACG	348
400	GSTP1 (Fwd)	P	fluor- (C*)GCGAT(T*)T(C-quencher)GGGGA(T*)T(T*)TAGG-quencher/blocker	349
401	GSTP1 (Fwd)	P	fluor-CC(T*)AAAA(T*)C(C-quencher)CCGAAA(T*)CGC-quencher/blocker	350
402	APC (Fwd)	E	GAAGTAGTTGTGTAATTYGTTGG	351
403	APC (Fwd)	E	CACCCTAACRAACTACACC	352
404	APC (Fwd)	I	TGCGGATTAGGGCGTTTTTTATTTTC	353
405	APC (Fwd)	I	TACAACCACATATCGATCACGTACG	354
406	APC (Fwd)	P	fluor-GGAGTTCGTCGA(T*)TGG(T*)TGGG-quencher/blocker	355
407	APC (Fwd)	P	fluor-CCCAACCAA(T*)CGACGAAC(T*)CC-quencher/blocker	356
408	EYA4 (Fwd)	E	GAGTTTTTYGGAGGGTTATAG	357
409	EYA4 (Fwd)	E	CAAACTACAAAAAACATTCAATCC	358
410	EYA4 (Fwd)	I	GCGTTTGGGTTTTTTCGGTGTC	359
411	EYA4 (Fwd)	I	ATCGCCGCAATTAAAAAACCCG	360
412	EYA4 (Fwd)	P	fluor-GGTTCGCGTTTTAAT(T*)TTTAGG(T*)ATTG-quencher/blocker	361
413	EYA4 (Fwd)	P	fluor-CAATACC(T*)AAAAAT(T*)AAAACGCGAACC-quencher/blocker	362
414	OLIG2(Fwd)	E	GTTATGGATTYGGAYGTTAG	363
415	OLIG2(Fwd)	E	CTCCRACRAACAATCACTC	364
416	OLIG2(Fwd)	I	GTTTGGTGTTTAG(T*)CGTTCGTC	365
417	OLIG2(Fwd)	I	CACTCGAAATAAA(C**)GAAAACACG	366
418	OLIG2(Fwd)	P	fluor-GGTAGTAGCGG(T*)AGCGTT(T*)TTATTG-quencher/blocker	367
419	OLIG2(Fwd)	P	fluor-CAATAAAAACGC(T*)ACCGC(T*)ACTACC-quencher/blocker	368
420	ADAMTS12(Fwd)	E	GGYGTAGTTTATTTYGGTT	369
421	ADAMTS12(Fwd)	E	ATTTAACCRACTCRACCAAC	370
422	ADAMTS12(Fwd)	I	GTATGTTTCGCGGTTTCGTAGTTC	371
423	ADAMTS12(Fwd)	I	ACTAAACCTAACG(T*)TCGAAACG	372
424	ADAMTS12(Fwd)	P	fluor- (C*)GTTCGTTCGG(T-quencher)G(T*)ATTTTTT(T*)TTCGG-quencher/blocker	373
425	ADAMTS12(Fwd)	P	fluor-CCGAAAAAAAAA(T-quencher)A(C**)ACCGAA(C**)GAAC-quencher/blocker	374
426	POU4F1(Fwd)	E	GTTTGAGTTGTTTTGATTTTAGTG	375

427	POU4F1(Fwd)	E	CTCCAACCTCAACTCTAAAC	376
428	POU4F1(Fwd)	I	GATTTTAGTGTCGCGTATTTTGGTTC	377
429	POU4F1(Fwd)	I	CTAAACTAAATCCCGCGAACCTCG	378
430	POU4F1(Fwd)	P	fluor-GGTTTTAT(T*)GGGGGTT(N*)AT(T*)TCGGGTAG-quencher/blocker	379
431	POU4F1(Fwd)	P	fluor-CTACCCGAAATAACCC(C**)CAA(N*)TAAAA(C**)C-quencher/blocker	380
432	ABCB1(Fwd)	E	GGTTTTTAGTATTTTTAYGAAGGT	381
433	ABCB1(Fwd)	E	CRATACRAAAACCTACTCTCTA	382
434	ABCB1(Fwd)	I	TTTGGATTTTGTTCGTCGTTAGTGC	383
435	ABCB1(Fwd)	I	CTACTCTCTAAACCCGCGAACG	384
436	ABCB1(Fwd)	P	fluor-GGTTTTAGTCG(T*)CGCGGACGATGT-quencher/blocker	385
437	ABCB1(Fwd)	P	fluor-ACATCGTCCGCGACGAC(T*)AAAACC-quencher/blocker	386
438	SOX17	I	GAGTTAGATTTAGAGCGCGTTGTTC	387
439	TAC1	I	GAGCGCGTTCGGATTTTTTTTTC	388

[0544] It is noted that these primers and probes identify the locations of various fluorophores and quenchers. However, it will be recognized that the particular fluorophores and quenchers are illustrative and not limiting and numerous amplification and/or detection strategies can be employed in the cartridges described herein. Accordingly, in various embodiments the methods and devices described herein can employ many different nucleic acid hybridization probes. Typically, for signal generation, the probes utilize a change in the fluorescence of a fluorophore due to a change in its interaction with another molecule or moiety brought about by changing the distance between the fluorophore and the interacting molecule or moiety. Alternatively, other methods of detecting a polynucleotide in a sample, including, but not limited to, the use of radioactively-labeled probes, are contemplated.

[0545] Fluorescence-based assays typically rely for signal generation on fluorescence resonance energy transfer, or "FRET", according to which a change in fluorescence is caused by a change in the distance separating a first fluorophore from an interacting resonance energy acceptor, either another fluorophore or a quencher. Combinations of a fluorophore and an interacting molecule or moiety, including quenching molecules or moieties, are known as "FRET pairs." The mechanism of FRET-pair interaction typically requires that the absorption spectrum of one member of the pair overlaps the emission spectrum of the other member, the first fluorophore. If the interacting molecule or moiety is a quencher, its absorption spectrum typically overlaps the emission spectrum of the fluorophore (*see, e.g.*, Stryer (1978) *Ann. Rev. Biochem.* 47: 819-846; Selvin (1995) *Meth. Enzymol.* 246: 300-335; and the like). Efficient FRET interaction is typically achieved when the absorption and emission spectra of the pair have a large degree of overlap. The efficiency of FRET interaction is linearly proportional to that overlap. Typically, a large magnitude of signal (*i.e.*, a high degree of overlap) is desired. FRET pairs, including fluorophore-quencher pairs, are therefore typically chosen on that basis.

[0546] A variety of labeled nucleic acid hybridization probes and detection assays that utilize FRET and FRET pairs are known. One such scheme is described by Cardullo *et al.*(1988) *Proc. Natl. Acad. Sci. USA,* 85: 8790-8794and in Heller *et al.* EP 0070685. It uses a probe comprising a pair of oligodeoxynucleotides complementary to contiguous regions of a target DNA strand. One probe molecule contains a fluorescent label, a fluorophore, on its 5' end, and the other probe molecule contains a different fluorescent label, also a fluorophore, on its 3' end. When the probe is hybridized to the target sequence, the two labels are brought very close to each other. When the sample is stimulated by light of an appropriate frequency, fluorescence resonance energy transfer from one label to the other occurs. FRET produces a measurable change in spectral response from the labels, signaling the presence of targets. One label could be a "quencher," which can be, *inter alia,* an interactive moiety (or molecule) that releases the accepted energy as heat.

[0547] Another type of nucleic acid hybridization probe assay utilizing a FRET pair is the "TaqMan®" assay described in Gelfand *et al.*U.S. Pat. No. 5,210,015, and Livak *et al.* U.S. Pat. No. 5,538,848. The probe is typically a a single-stranded oligonucleotide labeled with a FRET pair. In a TaqMan® assay, a DNA polymerase releases single or multiple nucleotides by cleavage of the oligonucleotide probe when it is hybridized to a target strand. That release provides a way to separate the quencher label and the fluorophore label of the FRET pair.

[0548] In certain embodiments non-FRET fluorescent probes, such as those described in, *e.g.*, Tyagi *et al.*, U.S. Pat. No. 6,150,097 can also be used. For example, the Tiyagi *et al.* patent describes how changes in the absorption spectra of the label pair can be used as a detectable signal as an alternative to change in fluorescence. When change in absorption is utilized, the label pair may include any two chromophores, that is, fluorophores, quenchers and other chromophores. The label pair may even be identical chromophores.

[0549] In some embodiments, dyes and other moieties, such as quenchers, are introduced into primers and/or probes used in the methods and cartridges described herein. In certain embodiments such dyes and quenchers include, but are not limited to dyes (fluors) suitable for use as FRET probes. In certain embodiments the dyes and/or quenchers comprise modified nucleotides. A "modified nucleotide" refers to a nucleotide that has been chemically modified, but still functions as a nucleotide. In some embodiments, the modified nucleotide has a chemical moiety, such as a dye or quencher, covalently attached, and can be introduced into a polynucleotide, for example, by way of solid phase synthesis of the polynucleotide. In some embodiments, the modified nucleotide includes one or more reactive groups that can react with a dye or quencher before, during, or after incorporation of the modified nucleotide into the nucleic acid. In some embodiments, the modified nucleotide is an amine-modified nucleotide, *i.e.*, a nucleotide that has been modified to have a reactive amine group. In some embodiments, the modified nucleotide comprises a modified base moiety, such as uridine, adenosine, guanosine, and/or cytosine. In some embodiments, the amine-modified nucleotide is selected from 5-(3-aminoallyl)-UTP; 8-[(4-amino)butyl]-amino-ATP and 8-[(6-amino)butyl]-amino-ATP; N6-(4-amino)butyl-ATP, N6-(6-amino)butyl-ATP, N4-[2,2-oxy-bis-(ethylamine)]-CTP; N6-(6-Amino)hexyl-ATP; 8-[(6-Amino)hexyl]-amino-ATP; 5-propargylamino-CTP, 5-propargylamino-UTP. In some embodiments, nucleotides with different nucleobase moieties are similarly modified, for example, 5-(3-aminoallyl)-GTP instead of 5-(3-aminoallyl)-UTP. Many amine modified nucleotides are commercially available from, *e.g.*, Applied Biosystems, Sigma, Jena Bioscience and TriLink. An illustrative, but non-limiting list of suitable fluors is shown in Table 6.

Table 6. Illustrative, but non-limiting fluorophores (fluorescent labels) for use in the primers and/or probes described herein.

Dye	Absorbance Wavelength	Emission Wavelength
Alexa fluor	345	442
Alexa fluor 430	430	545
Alexa fluor 488	494	517
Alexa fluor 532	530	555
Alexa fluor 546	556	573
Alexa fluor 555	556	573
Alexa fluor 568	578	603
Alexa fluor 594	590	617

Alexa fluor 633	621	639
Alexa fluor 633	650	668
Alexa fluor 660	663	690
Alexa fluor 680	679	702
Allophycocyanin	650	660
Aminocoumarin	350	445
Cy2	490	510
Cy3	550	570
Cy3.5 581	581	596
Cy5	650	670
Cy5.5	675	694
Cy7	743	770
FAM	495	516
Fluorescein FITC	495	518
HEX	535	556
Hydroxycoumarin	325	386
Methoxycoumarin	360	410
Red 613	480;565	613
Rhodamine Red-X	560	580
Rox	575	602
R-phycoerythrin (PE)	480;565	578
Tamara	565	580
Texas Red	615	615
TRITC	547	572
TruRed	490;675	695

[0550] If the assay is designed to detect one target DNA sequence then only one fluorescent hybridization probe needs to be used and, in certain embodiments, FAM, TET, or HEX (or one of their alternatives listed in Table 7) will be a good fluorophore to label the probe. These fluorophores can readily be excited and detected in various spectrofluorometric thermal cyclers. In addition, because of the availability of phosphoramidites derivatives of these fluorophores and the availability of quencher-linked control- pore glass columns, fluorescent hybridization probes with these labels can be entirely synthesized in an automated DNA synthesis process, with the advantage of relatively less expensive and less labor intensive probe manufacture.

Table 7. Additional illustrative fluorophore labels for fluorescent hybridization probes.

Fluorophore	Alternative Fluorophore	Excitation	Emission

		(nm)	(nm)
Cy3[3]	NED[2], Quasar 570[1], Oyster 556[4]	550	570
Cy5[3]	LC red 670[5], Quasar 670[1], Oyster 645[4]	650	670
HEX	JOE, VIC [B], CAL Fluor Orange 560[1]	535	555
LC red 640[5]	CAL Fluor Red 635 [A]	625	640
LC red 705[5]	Cy5.5[3]	680	710
ROX	LC red 610[5], CAL Fluor Red 610[1]	575	605
TET	CAL Fluor Gold 540[1]	525	540
Texas red	LC red 610[5], CAL Fluor Red 610[1]	585	605
TMR	CAL Fluor Red 590[1]	555	575
[1)]CAL and Quasar fluorophores are available from Biosearch Technologies; [2)]VIC and NED are available from Applied Biosystems; [3)]Cy dyes are available from Amersham Biosciences; [4)]Oyster fluorophores are available from Integrated DNA Technologies; and [5)]LC (Light Cycler) fluorophores are available from Roche Applied Science.			

[0551] In certain embodiments, multiple target genes are detected in a single multiplex reaction. In some embodiments, each probe that is targeted to a different gene is spectrally distinguishable (detectably different) from the other probes utilized in the multiplex reaction. Probe combinations suitable for multiplex detection are known to those of skill in the art. For example, illustrative combinations of detectably different fluorphores in four target multiplex systems include, but are not limited to:

[0552] 1) FAM, TMR, Texas red, and Cy5;

[0553] 2) FAM, TET, TMR, and Texas Red;

[0554] 3) FAM, HEX, Texas red, and Cy5; and

[0555] 4) FAM, Cy3, Texas red, and Cy5.

[0556] An illustrative combination of detectably different fluorphores in a five target multiplex systems is FAM, TET, TMR, Texas Red, and Cy5. Illustrative combinations of detectable different fluorophores in a six target multiplex system include, but are not limited to:

[0557] 1) FAM, TET, HEX, TMR, ROX, and Texas red; and

[0558] 2) FAM, HEX, LC red 610, LC red 640, LC red 670, and LC red 705.

[0559] It will be recognized that these combinations of fluorophores are illustrative and non-limiting and numerous other fluorophores will be available to those of skill in the art.

[0560] As noted above, for the design of fluorescent hybridization probes that utilize fluorescence resonance energy transfer (FRET), fluorophore-quencher pairs that have sufficient spectral overlap should be chosen. Fluorophores with an emission maximum between 500 and 550 nm, such as FAM, TET and HEX, are best quenched by quenchers with absorption maxima between 450 and 550 nm, such as dabcyl, BHQ-1, and the like (*see, e.g.*, Table 8 for illustrative quencher labels). Fluorophores with an emission maximum above 550 nm, such as rhodamines (including TMR, ROX and Texas red) and Cy dyes (including Cy3 and Cy5) are effectively quenched by quenchers with absorption maxima above 550 nm (including BHQ-2).

[0561] For the design of fluorescent hybridization probes that utilize contact quenching, any non-fluorescent quencher can serve as a good acceptor of energy from the fluorophore. For example, Cy3 and Cy5 are effectively quenched by the BHQ-1 and BHQ-2 quenchers.

Table 8. Illustrative quencher labels for fluorescent hybridization probes.

Quencher	Absorption Maximum (nm)
BHQ-1[4]	534
BHQ-2[4]	580
BHQ-3[4]	670
Dabcyl	475
DDQ-I[1]	430
DDQ-II[1]	630
Eclipse[2]	530
Iowa Black FQ[3]	532
Iowa Black RQ[3]	645
QSY-21[5]	660
QSY-7[5]	571
[1])DDQ or Deep Dark Quenchers are available from Eurogentec; [2])Eclipse quenchers are available from Epoch Biosciences; [3])Iowa quenchers are available from Integrated DNA Technologies; [4])BHQ or Black Hole quenchers are available from Biosearch Technologies; and [5])QSY quenchers are available from Molecular Probes.	

[0562] In certain embodiments nucleotides can quench the fluorescence of fluorophores, with guanosine being the most efficient quencher, followed by adenosine, cytidine and thymidine. In general, fluorophores with an excitation wavelength between 500 and 550 nm are quenched more efficiently by nucleotides than fluorophores with longer excitation wavelengths. In designing fluorescent hybridization probes, it can be desirable to avoid placing a fluorophore label directly next to a guanosine, to ensure higher fluorescence signals from the fluorophore.

[0563] The stabilizing effect of some fluorophore-quencher pairs that interact by contact quenching can have important consequences for the design of hybridization probes (*see, e.g.,* Marras *et al.* (2002) *Nucleic Acids Res.* 30: e122; Johansson *et al.*(2002) *J. Am. Chem. Soc.* 124: 6950-6956). For example, it has been observed that hybridization probes labeled with a fluorophore quenched by either BHQ-1 or BHQ-2 show an increase in hybrid melting temperature of about 4°C, compared to hybridization probes with the same probe sequence, but labeled with fluorophores quenched by dabcyl. It is also noted that strong affinity has been observed between the Cy dyes, Cy3 and Cy5, and the Black Hole quenchers, BHQ-1 and BHQ-2.

[0564] In view of the foregoing and the Examples and teachings provided herein, numerous primer/probe combinations will be available for use in the methods and cartridges described herein.

Cartridge, modules, and systems for DNA methylation analysis.

[0565] In certain embodiments cartridges are provided for performing the methods described herein (*e.g.*, determination of DNA methylation and, optionally RNA expression). In certain embodiments the cartridge comprises a column comprising a first matrix material, a sample receiving chamber, a temperature controlled channel or chamber, a plurality of chambers containing reagents and/or buffers, and when in use at least one of said chambers contains a DNA conversion reagent (*e.g.*, DABSO and/or a bisulfite reagent), and at least one of said chambers contains a desulphonation/elution buffer, and wherein said cartridge optionally comprises a second column comprising said second matrix material. In certain embodiments the cartridge is configured so that in use, the cartridge comprises a chamber containing a reagent comprising guanidinium thiocyanate ethanol (GTC-EtOH). In certain embodiments the second column is absent, while in other embodiments the second column is present. In certain embodiments the temperature controlled channel or chamber can simply be a heating channel or chamber, or it can be a thermocycling channel or chamber. In certain embodiments the cartridge further comprises a second heating channel or chamber (*e.g.*, a second thermocycling channel or chamber). In certain embodiments the cartridge is configured so that a DNA conversion step (*e.g.*, bisulfite incubation) and/or a desulphonation step occurs in the same reaction tube or chamber in which one or more PCR reactions are later performed.

[0566] In certain embodiments the bisulfite reagent is provided as a component of the cartridge. In certain other embodiments the cartridge is configured for the bisulfite reagent to be added to the cartridge at or near the time the sample is placed in the cartridge. In certain instances, the bisulfite reagent is added directly into a chamber in the cartridge, while in other embodiments, the bisulfite reagent is introduced into a loading port on the cartridge (*e.g.*, an injection port) to introduce the bisulfite reagent itno the cartridge. In certain embodiments the bisulfite reagent is introduced into the cartridge by the system operating the cartridge (*e.g.*, a processing module) while the cartridge is operating to determine DNA methylation.

[0567] In certain embodiments the reagent comprising guanidinium thiocyanate (*e.g.*, GTC-EtOH) is provided as a component of the cartridge. In certain other embodiments the cartridge is configured for the reagent comprising guanidinium thiocyanate to be added to the cartridge at or near the time the sample is placed in the cartridge. In certain instances, the reagent comprising guanidinium thiocyanate is added directly into a chamber in the cartridge, while in other embodiments, the reagent comprising guanidinium thiocyanate is introduced into a loading port on the cartridge (*e.g.*, an injection port) to introduce the bisulfite reagent itno the cartridge. In certain embodiments the reagent comprising guanidinium thiocyanate is introduced into the cartridge by the system operating the cartridge (*e.g.*, a processing module) while the cartridge is operating to determine DNA methylation.

[0568] In various illustrative, but non-limiting embodiments, the conversion reagent (*e.g.*, bisulfite reagent) comprises a compound selected from the group consisting of sodium metabisulfite, potassium bisulfite, cesium bisulfite, DABSO, and ammonium bisulfite. In certain embodiments the bisulfite is provided in a reagent mix comprising scavengers (*e.g.*, Trolox, hydroquinone, *etc.*) to prevent sulfite oxidation and/or catalysts. In certain embodiments the bisulfite is provided in a reagent mix comprising polyamines as catalysts.

[0569] In various embodiments the first matrix material and/or said second matrix material, when present, comprises a material selected from the group consisting of glass or silica, an ion exchange resin, and hydroxyapatite.

[0570] In various embodiments the cartridge comprises one or more chambers (*e.g.*, 1 chamber, 2 chambers, 3 chambers, 4 chambers, *etc.*) each containing one or more reagents selected from the group consisting of methylation specific PCR primers, methylation specific PCR probes, PCR enzyme(s) (*e.g.*, polymerase), reverse transcriptase, and PCR reaction buffer.

[0571] In certain embodiments the cartridge contains one or more chambers containing primers specific for bisulfite-converted methylated and/or unmethylated sequences. In certain embodiments the cartridge comprises one or more chambers containing primers and probes for a MethyLight PCR protocol. In certain embodiments the cartridge comprises one or more chambers containing reagents for TaqMan PCR reactions. In certain embodiments the cartridge comprises one or more chambers containing one or more fluorescent probes that are markers for amplified methylated sequences and/or one or more fluorescent probes that are markers for amplified unmethylated sequences. In certain embodiments the probes comprise a fluorescent reporter dye and a quencher dye, where the probes provides a signal upon cleavage by the 5' to 3' nuclease activity of Taq DNA polymerase. In certain embodiments the cartridge comprises a plurality of probes each specific to a different methylated region in an amplified region of interest. In certain embodiments the cartridge comprises a single probe specific to a methylated region in an amplified region of interest. In certain embodiments the cartridge comprises a plurality of probes each specific to the same methylated region in an amplified region of interest.

[0572] Illustrative primers and probes include, but are not limited to primers and/or probes to determine methylation of a promoter region of a gene selected from the group consisting of *APC, ARF, CDKN2B, CDKN2A, BRCA1, VLH, hMLH1, MGMT. RASSF1A, ADAMTS1, BNC1, HIST1H3C, HOXB4, RASGRF2, TM6SF1, AKR1B1, HIST1H4F, PCDHGB6, NPBWR1, ALX1*, and *HOXA9*. In certain embodiments the primers and/or probes are selected to determine methylation of a promoter region of a gene selected from the group consisting of *MGMT, RASSF1A, ADAMTS1, BNC1, HIST1H3C, HOXB4, RASGRF2, TM6SF1*, and *AKR1B1*. In various embodiments the PCR primers, and/or probes, and/or enzymes are provided as beads, *e.g.*, as described in U.S Patent Publication No: 2006/0068399, which is incorporated herein by reference for the beads and bead formulations described therein.

[0573] In various embodiments the cartridge is configured so that the sample receiving chamber, said column(s), the plurality of chambers, and the temperature controlled channel or chamber, are selectively in fluid communication. In certain embodiments the selective fluid communication is provided by microfluidic channels and valves. In certain embodiments the seelctive fluid communction is provided by providing the sample receiving chamber, said column(s), said plurality of chambers, the heating channel or chamber or a port into the heating channel or chamber, disposed around a central valve and selectively in fluid communication with a channel in said central valve.

[0574] In certain embodiments the cartridge is configured so that, when in use, the cartridge comprises: a first chamber containing a sample; a second chamber containing a guanidinium thiosulfate-ethanol (GTC-EtOH) solution; a third chamber containing a bisulfite reagent; a fourth chamber containing a buffer; a fifth chamber containing a rinse solution; and a sixth chamber containing an elution/desulfonation reagent. In certain embodiments the cartridge comprises a seventh chamber containing PCR primers and/or probes and/or PCR enzymes. In certain embodiments the cartridge comprises an eighth chamber also containing PCR primers and/or probes and/or PCR enzymes.

[0575] Figures 1A, 1B and 2 illustrate one cartridge suitable for practice of the methods described herein. The illustrated cartridges are based on the GENEXPERT® cartridige (Cepheid, Inc., Sunnyvale, CA). As shown in Figure 2, panel A the cartridge **200** comprises a cartridge body **202** containing a plurality of reagent and/or buffer chambers **208**. The chambers are disposed around a central syringe barrel **206** that is in fluid communication with a valve body **210** (panel B and Figure 1B) and that is sealed with a gasket **204**. The valve body **210** can comprise a cap **212** and the entire cartridge body can be supported on a cartridge base **226**. A "plunger" not shown can be operated to draw fluid into the syringe barrel **206** and rotation of the syringe barrel **206** and asscoated valve body **212** provides selective fluid communication between the chambers **208** a cavity **214** that can contain a matrix material as described herein and function as a column. In various embodiments the cartridge further comprises one or more temperature controlled channels or chambers **216** that can, in certain embodiments, function as thermocycling chambers. The temperature controlled channels or chambers are also selectively in fluid communiction with the cavity **214** and/or the chambers **208**. As shown in Figure 1A, in certain embodiments, the cartridge provides optical windows to provide real-time detection of, *e.g.*, amplification products, base identity in sequencing operations, and the like.

[0576] In certain embodiments the cartridge **200** is configured for insertion into a reaction module **300**, *e.g.*, as shown in Figure 3A. As illustrated in Figure 3B the module is configured to receive the cartridge **200**. In certain embodiments the reaction module provides heating plates **308** to heat the temperature controlled chamber or channel. The module can optionally additionally include a fan **304** to provide cooling where the temperature controlled channel or chamber is a thermocycling channel or chamber. Electronic circuitry **302** can be provided to pass information (*e.g.*, optical information) top a computer for analysis. In certain embodiments the module can contain optical blocks **306** to provide excitation and/or detection of one or more (*e.g.*, 1, 2, 3, 4, or more) optical signals representing, *e.g.*, various nucleic acid targets. In various embodiments an electrical connector **312** can be provided for interfacing the module with a system (*e.g.* system cotroller or with a discrete analysis/controller unit. As illustrated, in Figure 3B the sample can be introduced into the cartridge using a pipette **310**.

[0577] In certain embodiments, the module also contains a controller that operates a plunger in the syringe barrel and the rotation of the valve body.

[0578] In certain embodiments a system (*e.g.*, a processing unit) is provided. One illustrative, but non-limiting embodiment is shown in Figure 3C. In certain embodiments, the processing unit comprises an enclosure configured to contain one or more sample processing modules where each processing module is configured to hold and operate a removable cartridge as described herein. In certain embodiments the system is configured to operate the sample processing modules to perform sample processing to determine methylation of one or more target nucleic acids and optionally to determine the level of one or more target RNA/DNA sequences within a corresponding removable sample cartridge, wherein the processing on a sample within the corresponding removable sample cartridge performs a method as described herein. In certain embodiments the system is configured to contain one sample processing module. In certain embodiments the system is configured to contain at least two sample processing modules, or at least 4 sample processing modules, or at least 8 sample processing modules, or at least 12 sample processing modules, or at least 16 sample processing modules, or at least 20 sample processing modules, or at least 24 sample processing modules, or at least 28 sample processing modules, or at least 32 sample processing modules, or at least 64 sample processing modules, or at least 128 sample processing modules. In certain embodiments the system provides a user interface that allows the user input operational instructions and/or to monitor operation of the cartridges to determine DNA methylation.

[0579] While the methods described herein are described primarily with reference to the GENEXPERT® cartridge by Cepheid Inc. (Sunnyvale, CA) (*see, e.g.*, Figure 1A), it will be recognized, that in view of the teachings provided herein the methods can be implemented on other cartridge/microfluidic systems. Such cartridge/microfluidic systems can include, for example microfluidic systems implemented using soft lithography, micro/nano-fabricated microfluidic systems implemented using hard lithography, and the like.

High volume sample preparation (HVSP) cartridge.

[0580] In various embodiments cartridges are provided for the preparation of large sample volumes. In certain embodiments the sample preparation cartridges comprises GENEXPERT® cartridges modified for high volume sample preparation (*e.g.*, as shown in Figure 20). In certain embodiments, *e.g.*, when the cartridge is based on a GENEXPERT® cartridge comprises one or more channels or chambers comprising an affinity matrix that binds DNA, a plurality of chambers disposed around a central valve assembly and selectively in fluid communication with said central valve assembly where the central valve assembly is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with the central valve wherein said plurality of chambers comprises at least two different chambers each configured to receive up to about 4 ml (or up to about 5 ml) of sample solution (in certain embodiments chamber 2 has a maximum volume of about 4 ml, while chamber 3 has a maximum volume of about 4.5 ml), a chamber containing PEG (*e.g.*, PEG200), a chamber containing an alkaline solution (*e.g.*, KOH solution), and a chamber containing a buffer (*e.g.*, Tris). In certain embodiments the plurality of chambers comprises at least three different chambers each configured to receive up to about 4 ml (or up t about 5 ml) of sample solution. In certain embodiments the plurality of chambers comprises a chamber containing a wash solution (*e.g.*, GTC-ethanol wash solution which is typically 1.25M guanidinium thiocyanate, 25 mM Tris pH 7.0, 50% ethanol). In certain embodiments the cartridge comprises a chamber configured for removal of a processed sample. In certain embodiments the sample chambers, when in use, contain sample solution, GTC and alcohol(*e.g.*, isopropanol). In certain embodiments the sample chambers, when in use contain sample solution, GTC and alcohol in substantially equal volumes. In certain embodiments the cartridge, when in use, comprises 4 ml of sample solution GTC and isopropanol disposed in each of said chambers configured to receive a sample. In certain embodiments the cartridge provides DNA or RNA recovery that is substantially linear with respect to the sample volume between 0.5 ml and about 4 ml of sample.

[0581] In certain embodiments the HVSP cartridge is configured to perform a DNA conversion (*e.g.*, bisulfite conversion) to provide a methylation analysis. Accordingly in certain embodiments, the HVSP cartridge is configured to contain, or to receive immediately or shortly prior to use, a conversion reagent (*e.g.* a bisulfite reagent, DABSO, *etc.*). In certain embodiments, the HVSP cartridge can be configured to also contain reagents for and to provide a desulphonation of converted DNA. Alternatively, in certain embodiments, the conversion is performed in the HSVP cartridge while the desulphonation and methylation analysis (*e.g.*, PCR) is performed in the second cartridge (*e.g.*, as illustrated in the work flows shown in Figure 20B).

cfDNA Sample Preparation cartridge.

[0582] In certain embodiments a sample preparation cartridge is provided that is particular well suited to the preparation (and optional analysis) of nucleic acids from plasma or serum is provide. One illustrative, but non-limiting embodiment is shown in Figure 17. As illustrated therein in certain embodiments the cartridge comprises a channel or chamber comprising an affinity matrix that binds DNA, a plurality of chambers disposed around a central valve assembly and selectively in fluid communication with the central valve assembly where the central valve assembly is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with the central valve where the plurality of chambers comprises: a chamber configured to receive up to about 5 ml or up to about 4 ml of sample solution; a chamber containing PEG (*e.g.*, PEG200); a chamber containing GTC-EtOH; a chamber containing an alkaline solution (*e.g.*, KOH); and a chamber containing a buffer (*e.g.*, Tris). In certain embodiments the plurality of chambers further comprises a chamber containing a conversion reagent (*e.g.*, a bisulfite reagent). In certain embodiments the plurality of chambers comprises a chamber containing a wash solution (*e.g.*, GTC-ethanol wash (typically 1.25M guanidinium thiocyanate, 25 mM Tris pH 7.0, 50% ethanol)). In certain embodiments the plurality of chambers comprises a chamber containing beads comprising one or more PCR primers and/or probes. In certain embodiments the chamber containing PEG contains about 1 ml of PEG. In certain embodiments the chamber containing an alkaline solution contains about 500 μL of solution. In certain embodiments the chamber containing GTC-EtOH contains about 2 ml GTC-EtOH. In certain embodiments the chamber containing a buffer contains about 2 mL of buffer.

[0583] It will be recognized that this configuration is illustrative, and using the teaching provided herein numerous other preparation cartridge configurations will be available to one of skill in the art.

Use of DABSO as an alternative to bisulfite

[0584] It was a surprising discovery that DABSO can be used to perform a conversion of DNA in a manner analogous to the use of bisulfites for the conversion of DNA and detection of methylation. Accordingly, in certain embodiments, methods of utilizing DABSO to converting cytosine residues in a DNA to uracil, while leaving 5-methylcytosine residues substantially unaffected are provided. In certain embodiments the methods involve contacting a sample comprising DNA with DABSO to convert the DNA, and desulfonating the converted DNA, to produce a DNA in which cytosine residues are converted to uracil, but 5-methylcytosine residues substantially unaffected. In certain embodiments the DABSO is provided at a concentration ranging from about 2 M up to about 5 M. In certain embodiments the DABSO is provided at a concentration of about 2.5 M. In certain embodiments the DABSO is dissolved in an alkaline aqueous solution (*e.g.*, a KOH solution). In certain embodiments the reagent comprising DABSO comprises DABSO dissolved in a solution comprising KOH.

[0585] In certain embodiments the methods involve heating the DABSO/DNA solution to a temperature ranging from about 55°C to about 90°C. In certain embodiments the DABSO is reacted with the DNA for a period of time ranging from about 15 minutes up to about 90 minutes. After the DNA is converted, it is desulphonated (*e.g.*, by contacting the converted DNA with an alkaline reagent (*e.g.*, KOH solution). In certain embodiments the conversion and/or desulphonation is performed on the DNA bound to a column, while in other embodiments the conversion and/or desulphonation is performed on the DNA in solution.

[0586] Also provided are methods of analyzing DNA methylation, where the methods involve providing a DNA sample, converting DNA in the sample using a DABSO reagent, *e.g.*, as described above, and performing methylation specific PCR and/or nucleic acid sequencing, and/or high resolution melting analysis (HRM) on the converted nucleic acid to determine the methylation of said nucleic acid. In certain embodiments the providing of a DNA sample comprises preparing a sample as described herein (*e.g.*, using lysis solutions and/or preparation cartridges as described herein.

Kits.

Kits for methylation detection.

[0587] In certain embodiments kits are provided for performing the methods described herein. In one illustrative embodiment, the kits comprise a container containing a reaction cartridge as described herein, a container containing a sample processing reagent as described herein, and a container containing a conversion reagent (*e.g.*, a bisulfite reagent) as described herein. In certain embodiments the bisulfite reagent is provided in a chamber of the cartridge. In certain embodiments the bisulfite reagent is provided in a container separate from the cartridge. In certain embodiments, the sample processing reagent is provided in a chamber of the cartridge. In certain embodiments, particularly where the sample processing reagent comprises guanidinium thiocyanate the sample processing reagent is provided in a container separate from the cartridge.

[0588] In addition, the kits optionally include labeling and/or instructional materials providing directions (*i.e.*, protocols) for the use of the cartridges described herein to determine DNA methylation and, optionally, RNA expression.

[0589] In certain embodiments a kit for the determination of DNA methylation is provided where the kit comprises a container containing a cartridge for determining the methylation state of a nucleic acid as described herein. In certain embodiments the kit further comprises a lysis solution as described herein (*e.g.*, a lysis solution for serum or plasma, *e.g.*, as described in Table 11, and/or a lysis solution for FFPE samples, *e.g.*, as described in Table 12). In certain embodiments the kit comprises a container containing proteinase K. In certain embodiments the kit contains a conversion reagent (*e.g.*, a bisulfite reagent) in the cartridge or in a container separate from the cartridge. In certain embodiments the separate container can contain a pre-measured volume of conversion reagent suitable for one "run" of the cartridge. In certain embodiments the conversion reagent comprises a compound selected from the group consisting of sodium metabisulfite, potassium bisulfite, cesium bisulfite, ammonium bisulfite, and DABSO. In certain embodiments the kit comprises a container containing a sample processing reagent. In certain embodiments the sample processing reagent comprises guanidium thiocyanate and/or ethanol.

[0590] In various embodiments the kit can additionally contain a cartridge for sample preparation as described herein (*e.g.*, as illustrated in Figure 20).

[0591] In certain embodiments the kit contains instructional materials teaching the use of a cartridge for the determination of DNA methylation. Where a sample preparation cartridge is included in the kit the kit can additionally contain instructional materials teaching the use and operation of the sample preparation cartridge.

Kits for DABSO DNA conversion and methylation detection.

[0592] In certain embodiments kits are provided for the use use of DABSO as a conversion reagent, *e.g.*, in the detection of the methylation state of a DNA. In certain embodiments the kits comprise a container containing a conversion reagent comprising DABSO, and a container containing a desulphonation reagent. In certain embodiments the kit comprises a column comprising an affinity matrix (*e.g.*, a silica matrix material). In certain embodiments the kits comprise a container containing a binding buffer and/or a container containing an elution buffer. In certain embodiments the kit comprises a container containing a wash buffer.

[0593] In certain embodiments the kit further comprises a lysis solution as described herein (*e.g.*, a lysis solution for serum or plasma, *e.g.*, as described in Table 11, and/or a lysis solution for FFPE samples, *e.g.*, as described in Table 12). In certain embodiments the kit comprises a container containing proteinase K.

[0594] In various embodiments the kit can additionally contain a cartridge for sample preparation as described herein (*e.g.*, as illustrated in Figure 20).

[0595] In certain embodiments the kit contains instructional materials teaching the use of the kit to convert a nuclei acid for determination of the methylation state of the nucleic acid.

[0596] While the instructional materials in the kits described above typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

[0597] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

[0598] To validate the method human genomic DNA (HGDNA) was used as a starting sample to monitor sample preparation, bisulfite conversion, sample cleanup, and methylation specific qPCR in a Cepheid GENEXPERT® cartridge. In order to measure bisulfite conversion efficiency, half of the DNA-bisulfite mix was loaded and heated in the 50 µL cartridge tube during the bisulfite conversion step. Therefore, under optimal conversion conditions approximately half of the HGDNA is converted and the other half remains unconverted.

[0599] Primers and Taqman probes for the qPCR step were designed for one unconverted gene (*HMBS* (hydroxymethylbilane synthase housekeeping gene)) and one converted gene (*ACTB* (beta actin)), and the conversion efficiency was then quantitated by comparison of cycle threshold values (Cts). Both *ACTB* and *HMBS* are commonly used as single or low copy reference genes, and thus we expect similar copy numbers per ng of HGDNA.

[0600] A representative GENEXPERT® run from 300 ng of HGDNA is shown below in Figure 5, with the ACTB qPCR curve in green and the HMBS qPCR curve in blue. The qPCR reaction was run for 45 cycles with a 3 temperature cycle of 96 °C for 5 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds. At a manual threshold setting of 20 fluorescence units we observed a Ct of 31.7 for the converted ACTB gene and a Ct of 32.7 for the unconverted HMBS gene. Importantly, this result demonstrates that we are able to achieve near-optimal bisulfite conversion efficiency of HGDNA in our cartridge at physiological relevant concentrations of DNA found in FFPE tissue slices and plasma/serum samples. Further specificity for fully converted sequences can be achieved through a nested qPCR reaction or by heating the entire sample. However, neither option would be absolutely required for methylation specific qPCR in the GENEXPERT® because primer and probe sets are designed to amplify only the converted sequences. Thus remaining unconverted DNA sequences would act as carrier DNA, which notably is frequently added during bisulfite conversion, DNA isolation, and PCR methods.

Example 2

[0601] Figures 6A and 6B show the linearity of converted ACTB. In particular, Fig. 6A shows the results of a 15 cycle nested qPCR for ACTB using hgDNA. As can be seen from the panel on the right the signal (Ct value) is substantially linear between about 25,000 copies and about 100 copies. Fig. 6B shows the results of a 20 cycle nested qPCR for ACTB using hgDNA. These plots demonstrate the sensitivity of the cartridge for hgDNA . Dropouts start occurring around 20-50 copies with a sensitivity of about 25 copies of converted DNA.

[0602] Figures 7A, 7B, and 7C show the results of qPCR for six methylated targets (AKR1B1, HOXB4, TM6SF1, RASGRF2, and RASSF1A). Fig. 7A show the results of 20 cycle nested qPCR for controls (25 ng of HSDNA, and 5000 MBA-453 cells whose DNA is not bisulfite-converted). Fig. 7B shows the results of 20 cycle nested qPCR for the six methylated targets using DNA from MBA-453 cells that has been bisulfite converted. A strong signal is shown for all targets. HIST1H3C was not reliably detected. Fig. 7C shows the results of 20 cycle nested qPCR for the six methylated targets using DNA from MBA-453 cells that has been bisulfite converted and is in a carrier comprising 1 μg of SS and 10 ng of HS DNA. Dropouts were observed at about 100 cells and below, however, with the carrier, there were significantly fewer dropouts.

Example 3

[0603] Figure 8 illustrates the results of a determination of conversion efficiency. The conversion efficiency is about 66% (~1 Ct) the difference between unconverted HMBS and converted ACTB. Ideal Ct with 100% binding/elution, 100% conversion, and 100% binding elution is about 24-25. The experiments appear to show a 50% binding/elution, 50-66% conversion, and 50% binding/elution for a 10-fold reduction and a Ct of about 27.

[0604] Figure 9 illustrates the increase in specificity for converted DNA produced by nested qPCR. Nested PCR appears to increase the specificity for converted DNA, to increase the specificity for methylated DNA and to reduce contamination issues.

[0605] Figure 10 illustrates the specificity of the methylation cartridge. No specificity is shown for unconverted DNA (top panel) or unmethylated DNA (bottom panel) except for HIST1H3C.

[0606] Figures 11A and 11B show some illustrative but non-limiting workflows for analysis of methylation using a cartridge (*e.g.*, a GENEXPERT® cartridge). Fig. 11A illustrates one work flow for analysis of DNA methylation in a serum sample. As illustrated in this workflow, serum is added to a lysis reagent vial and mixed/vortexed. The sample is then dispensed into a sample port in the cartridge. The cartridge is placed in the system for analysis.

[0607] Fig. 11A illustrates one work flow for analysis of DNA methylation in a tissue section (*e.g.*, frozen or formalin-fixed paraffin embedded (FFPE) section). As shown therein, in one embodiment, a tissue section (*e.g.*, a 4 μm FFPE section) is provided. FFPE lysis reagents are added (*see, e.g.*, PCT/US2013/061863 (WO/2014/052551 for illustrative lysis reagents) and the mixture can be heated. Ethanol can be added and the mixture vortexed. The sample is then dispensed into a sample port in the cartridge. The cartridge is placed in the system for analysis.

[0608] Figure 12 illustrates the results for a FFPE cell button for converted ALU and methylated RASSF1A.

Example 4

Detection of Markers for Breast Cancer Monitoring

Materials and Methods:

[0609] Either 1000 MBA-453 cells or 25 ng of human sperm (HS) DNA were added to 2.5 mL of binding buffer (2.25 M Guanidinium thiocyanate, 22.5 mM Tris pH 7.0, 0.5% Tween20, 50% Ethanol, and 0.005% SE-15 antifoam). The 2.5 mL solution of cells or DNA was added to chamber 2 of the Cepheid methylation cartridge (layout in Figure 13A). The remaining chambers in the methylation cartridge were filled as follows: Chamber 3 – 3.2 mL of Wash buffer (1.25M Guanidinium thiocyanate, 25 mM Tris pH 7.0, 50% Ethanol), Chamber 4 – 90 μL of 7M Ammonium Bisulfite, Chamber 5 – 4 mL of 50 mM Tris pH 8.5, Chamber 8 – 1 mL of PEG200 Rinse, Chamber 9 – quantitative PCR beads including EZR (Taq) and TSR (6 target breast cancer multiplex for RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2, TM6SF1, *see* Table 9, below), Chamber 10 – 500 μL of 15 mM KOH, and Chamber 11 – nested beads including EZR (Taq) and TSR (6 target breast cancer multiplex for RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2, TM6SF1). The methylation cartridge was then loaded into a Cepheid GeneXpert and the entirety of the methylation assay was completed by the GeneXpert – the first DNA sample prep, the bisulfite conversion, the second post conversion DNA sample prep, the desulphonation, and the 20 cycle nested and quantitative PCR reactions.

[0610] A flow chart illustrating the methylation protocol is shown in Figure 13B. It is noted that the PEG200 was filled in the waste chamber 8, and after the assay starts the PEG200 is dispensed into Chamber 1. The PEG200 is a viscous liquid that cannot easily be directly loaded in the smaller chamber 1. Additionally, chamber 1 acts as an air chamber when the cartridge is first loaded before becoming the PEG200 chamber. Thus, the assay begins with Chamber 1=air and Chamber 8=PEG200 and is quickly switched to Chamber 1=PEG200 and Chamber 8=Waste after cartridge loading.

[0611] The numbers shown in the "Initial Vol." column of Figure 13A just refer to liquid volumes. In this case there are just 2xbeads in chamber 11- 1x TSR bead (primer and probes for the 6 targets) and 1x EZR bead (Phoenix Taq). These beads are for the final qPCR reaction. Similarly, there are 3x beads in chamber 9- 1xTSR bead (primers for the 6 targets), 1xTris bead (to quench KOH) and 1x EZR bead (Phoenix Taq). These beads are for the first 15-20 cycle PCR reaction.

[0612] It is also noted that Chamber 6 is an air chamber throughout the entire assay and is never filled. Chamber 7 is used as sort of a gateway to the PCR tube in the back of the cartridge. It is not filled to start the assay but is filled during the assay on 3 occasions before loading into the tube - 1) the DNA-bisulfite mix that is heated in the tube for conversion 2) the 15-20 cycle PCR reaction and 3) the final qPCR reaction.

[0613] The primers shown in the Table 9 provided shows five sequences for each gene – two extension primers and 2 qPCR primers for each nested amplification and one probe. The first 15-20 cycle PCR reaction was not specific for methylation but only the converted DNA sequences (*i.e.*, they do not cross CpGs and in a couple instances when they do we use an R= purine or Y= pyrimidine to catch both methylated and unmethylated). The second 45 cycle qPCR reaction contains both primers and probes that are specific for typically 2-3 methylated CpGs.

Results:

[0614] The methylation cartridge was run using 1000 MBA-453 cells with and without bisulfite (Figure 15A-15B) and 25 ng of HS DNA with bisulfite(Figure 15C) that was primarily unmethylated at each gene promoter with the exception of HIST1H3C. There was little or no amplification of any of the targets in either the no bisulfite or unmethylated HS DNA control reactions (Figure 15A, 15C). With the addition of bisulfite, the methylation cartridge picked up high levels of methylation at multiple gene promoters from 1000 MBA-453 cells, specifically AKR1B1, RASSF1A, HOXB4, and RASGRF2.

Table 9. Nested primers for *RASSF1A, AKR1B1, HOXB4, HIST1H3C, RASGRF2*, and *TM6SF1*. C*, T* are optionally functionalized (*e.g.*, to alter probe T_m) bases.

Gene/ Probe name	Type	SEQUENCE	SEQ ID

			NO
RASSF1A			
olAK61	ext primer	GTTTTATAGTT(T*)TTGTATTTAGG	62
olAK41	ext primer	AACTCAATAAACTCAAACTCCC	42
olAK1	qPCR primer	GCGTTGAAGTCGGGGTTC	2
olAK2	qPCR primer	CCCGTACTTCGCTAACTTTAAACG	3
olAK63	qPCR probe	fluor-(C*)TGGTTTCGT(T-quencher)CGGT(T*)CGCG-quencher/blocker	64
HIST1H3C			
olAK60	ext primer	GGATTTTTGAAATATTATAGGATTAATTAG	61
olAK43	ext primer	ATAAAATTTCTTCACRCCACC	44
olAK59	qPCR primer	TCGTACGAAGTAAATAGTTCGTAAG	60
olAK54	qPCR primer	CCGATAACCGAAACGCTCTTAC	56
olAK65	qPCR probe	fluor-CAAACTACTTACGCGAAACTT(T*)ACCGCC-quencher/blocker	66
RASGRF2			
olAK44	ext primer	GAGGGAGTTAGTTGGGTTAT	45
olAK45	ext primer	CCTCCAAAAAATACATACCC	46
olAK13	qPCR primer	GTAAGAAGACGGTCGAGGCG	14
olAK14	qPCR primer	ACAACTCTACTCGCCCTCGAA	15
olAK67	qPCR probe	fluor-AAACGAACCACTTCTCG(T*)ACCAACGAC-quencher/blocker	129
AKR1B1			
olAK58	ext	GYGTAATTAAT(T*)AGAAGGTTTTTT	59
olAK47	ext	AACACCTACCTTCCAAATAC	48
olAK19	qPCR	GCGCGTTAATCGTAGGCGTTT	20
olAK20	qPCR	CCCAATACGATACGACCTTAAC	12
olAK75	qPCR	fluor-(C*)A(C*)GCGTACCT(T-quencher)TAAA(T*)AACCCG(T*)AAAATCG-quencher/blocker	76
HOXB4			
olAK48	ext primer	TTAGAGGYGAGAGAGTAGTT	49
olAK49	ext primer	AAACTACTACTAACCRCCTC	50
olAK25	qPCR	CGGGATTTTGGGTTTTCGTCG	26

	primer		
olAK26	qPCR primer	CGACGAATAACGACGCAAAAAC	27
olAK76	qPCR probe	fluor-AACCGAACGA(T*)AACGAAA(N*)ACGACGAA-quencher/blocker	77
TM6SF1			
olAK50	ext primer	AGGAGATATYGTTGAGGGGA	51
olAK51	ext primer	TCACTCATACTAAACCRCCAA	52
olAK56	qPCR primer	GTTTAGCGGGATGCGGTG	57
olAK57	qPCR primer	ACACGAAAACCCCGATAAC	58
olAK77	qPCR probe	fluor-AAACACTCATCGCAACCGCCGCG-quencher/blocker	34

[0615] The primers shown in Table 9 are illustrative and not limiting. Numerous other primers and nested primer sets will be available to those of skill in the art. By way of example, illustrative primers for the detection of methylation of *ADAMTS1* and *BNC1* genes associated with pancreatic cancer and for the detection of methylation of the *MGMT* gene associated with glioma are shown in Table 10.

Table 10. Illustrative primers for the detection of methylation of *ADAMTS1* and *BNC1* genes associated with pancreatic cancer and for the detection of methylation of the *MGMT* gene associated with glioma.

Gene/ Probe name	**Type**	**Sequence**	**SEQ ID NO**
BNC1	external primer	CCCRCAAACCRCGAAAACCTC	227
	external primer	GTTTTTTTTYGGGAGAGGTAAATA	228
	qPCR internal primer	CCGACGACCGACG	235
	qPCR internal primer	GGGAGAGGTAAATATCGATAC	236
	qPCR probe	fluor-TGGYGGGGG(T*)AGA(T*)ATTTT-quencher/blocker	389
ADAMTS1	external primer	CRCCTCCRAAACTAAAACAAC	229
	external primer	GGGTTATTGTAAAGTTAGGGTG	230
	qPCR	CGCGAAAATTAATACCTAACG	237

	internal primer		
	qPCR internal primer	TTAGGGTGCGTTATCGGAC	238
	qPCR probe	fluor-TCTACTCAAAACTCTCCCCTCTCC-quencher/blocker	390
MGMT	external primer	GTTTT(T*)AGAAYG(T*)TTTGYGTTT	263
	external primer	AAAAAAC(T*)CCRCACTCTTCC	265
	qPCR internal primer	TTTCGACGTTCGTAGGTTTTCGC	266
	qPCR internal primer	GCACTCTTCCGAAAACGAAACG	267
	qPCR probe	fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC-quencher/blocker	268

Example 5

Sample Preparation for Plasma and FFPE Samples

[0616] Figure 17 illustrates one configuration of a cartridge that can be used to prepare DNA samples for PCR and/or methylation detection. The sample, obtained from serum or plasma, or an FFPE sample can simply be introduced into a sample chamber of the cartridge (*e.g.*, chamber 3) and operation of the cartridge as described herein provides a sample ready for PCR and/or methylation detection.

Sample preparation

[0617] In one illustrative, but non limiting embodiment, a serum or plasma sample is prepared (*e.g.*, for analysis of cfDNA) by treating the serum or plasma with proteinase K. Then the proteinase K treated serum/plasma is mixed with a lysis solution comprising guanidinium thiocyanate (GTC), buffer (*e.g.*, Tris pH 7.0), a detergent (*e.g.*, Tween 20), and an optional antifoam (*e.g.*, antifoam SE15). An alcohol (*e.g.*, isopropanol) is added to the solution which is then introduced into the cartridge for sample processing. In one embodiment the lysis solution is formulated as shown in Table 11. The proteinase K treated serum/plasma can be mixed with lysis solution and alcohol in a ratio corresponding to 1.3 mL proteinase K treated serum/plasma, 2.2 mL lysis solution, and 1.5 ml alcohol. In certain embodiments the serum/plasma sample is treated with proteinase K for about 15 minutes.

The lysis solution is added cold and held/mixed for about 10 minutes. Then isopropanol is added to the mixture which is then loaded into the cartridge for processing.

[0618] As noted above, for serum/plasma the alcohol (*e.g.*, isopropanol) precipitations are typically done at RT, and in particular typically not performed with "salty" solutions. In certain embodiments longer room temperature precipitation times can be used.

Table 11. Lysis solution for serum or plasma.

Reagent	Amount
Guanidine thiocyanate (GTC)	4.5M
Buffer (*e.g.*, Tris) pH 7.0	45 mM
Detergent (*e.g.*, Tween20)	1%
Antifoam SE15	0.01%

[0619] In another illustrative, but non-limiting embodiment, a formalin fixed paraffin-embedded (FFPE) sample is prepared by combining the FFPE sample with proteinase K and a lysis solution comprising a buffer (*e.g.*, HEPES), a chelator (*e.g.*, EDTA), NaCl, $MgCl_2$, and optionally sodium azide and/or an antifoaming agent. The solution is heated (*e.g.*, at 70°C to 90°C) for a period of time ranging, for example from about 10 minutes up to about 4 hours. An alcohol is added to the solution and the solution is then introduced into the cartridge for sample processing. In one embodiment the lysis solution is formulated as shown in Table 12. In one illustrative, but non-limiting embodiment, 1.2 mL of the lysis solution shown in Table 12 is added to the FFPE section(s). Proteinase K is added and the mixture is heated, *e.g.* at 80°C for about 15 minutes. In certain embodiments heating is performed at 56°C for 2 hours followed by 90°C for 30 minutes. Then 1.2 mL of ethanol is added to the mixture and the mixture is loaded into a sample chamber of the cartridge for processing.

Table 12. Lysis buffer for formalin fixed paraffin embedded (FFPE) sample.

Tween20	1%
NaCl	400mM
EDTA	25mM
$MgCl_2$	10mM
HEPES pH 7.2	50mM

Sodium Azide	0.01%
SE15	0.01%

Cartridge operation and extraction performance.

[0620] When cfDNA is being prepared, in certain embodiments, it is possible to include extraction controls to permit monitoring of the quality of the DNA preparation. As illustrated in Figure 18, there are two different bead sets. One bead set contains an endogenous HMBS primer and probe set for a SAC (sample assay control) and exogenous BG primer and probe set for a SPC (sample prep control). The other contains an endogenous Beta-Globin PP set for SAC (as well as BG SPC).

[0621] It was discovered, *inter alia*, that the use of GTC in the cartridge may be less important for serum than plasma samples. Without being bound by a particular theory it is believed that this may be due to the fact that serum contains less protein. Accordingly, in certain embodiments, the cartridge may contain less GTC or may omit GTC.

[0622] Figures 19A and 19B show a comparison of the results of cfDNA preparation performed using a cartridge as described herein compared to the results obtained using a conventional "tubefill" procedure. As illustrated in the qPCR results shown in Figure 19A, the binding and elution efficiencies obtained using the cartridge are extremely close (within one Ct) to those obtained using the tubefill protocol. As illustrated in Figure 19B titrations of sample concentrations show that the cartridge preparation is conservatively within 1 Ct of the tubefill preparation down to a sample concentration as low as about 10 pg. It is believe the cartridge preparation is even closer to the tubefill protocol at higher sample concentrations.

Example 6

Testing a High-Volume Sample Preparation Cartridge

[0623] In certain embodiments high volume sample preparation (HSVP) cartridges are provided for the preparation of large volumes of sample (*e.g.*, up to about 12 ml to 15 ml). This is particularly useful where the sample contains DNA at a low concentration (*e.g.*, cfDNA in serum or plasma). One such cartridge is schematically illustrated in Figure 20A. As shown therein the cartridge provides three chambers (chambers 2, 3, and 5) that can be used to receive a sample. In the illustrated embodiment, each of these chambers can receive about 4 mL of sample and, in certain embodiments, the sample comprises 4 mL of plasma/serum combined with 4 mL of GTC and 4 mL of alcohol (*e.g.*, isopropanol).

[0624] The sample is introduced into these chambers and the cartridge is operated as described herein to prepare the sample for PCR and/or methylation analysis. By way of illustration, in certain embodiments, operation of this cartridge can comprise binding DNA to an affinity column (*e.g.*, for cleanup) and eluting the DNA. In certain embodiments where a methylation analysis is to be performed, the operation of the cartridge can further comprise combining the DNA with a conversion reagent (*e.g.*, a bisulfite as described herein) and heating the mixture to convert the DNA. In certain embodiments, the HSVP cartridge can also be configured to desulphonates the converted DNA. In other embodiments, the DNA can be desulphonated in the second (*e.g.*, qPCR) cartridge as schematically illustrated in Figure 20B. The second cartridge can also perform the methylation analysis (*e.g.* a qPCR analysis).

[0625] Figure 21 shows a comparison of sample preparation results of DNA from plasma and serum between one cartridge and two cartridge protocols using the HMBS or β-globin primer and probe set. As shown therein, there was a linear increase in DNA recovery between 0.5 mL and 4 mL of serum or plasma. Moreover there was little to no loss when using one cartridge for the preparation and analysis or when using separate cartridges for preparation and analysis/

Example 7
Optimizing Bisulfite Conversion

[0626] In certain embodiments when using a cartridge for a methylation analysis as described herein one potential issue is the optimization of elution efficiency using the smallest volume possible. Small elution volumes are easier to deal with using spin columns. This problem can be addressed by using multiple heating steps to process larger sample volumes.

[0627] A second technical concern arises when heating a larger sample (*e.g.*, minimum 100 µL) when using a smaller (*e.g.*, 50 µL) heating tube or chamber. In certain instances, pressurizations between heating steps can make it difficult to reproducibly account for volume aspirates and dispenses. Secondly, the absence of pressurization can lead to to volume changes and bubbles especially at higher temperatures. Thirdly, it is possible to pick up air between heated and unheated samples during port changes in between heat steps.

[0628] To investigate these optimization of bisulfite conversion in a 50 µL tube using single and double heating steps was investigated. This experiment was performed as follows:

Pull 75-80 µL of bisulfite-DNA; heat 95°C-10s, 65°C-300s x8;

Pull rest + 5-10 µL; pressurize; heat 95°C-480s, 65°C-1800s x1.

[0629] The results for 0.5 mL of serum are shown in Figure 22 where the top panel is 1x Heat (converted 33.0, unconverted 34.4) N=4, and the bottom panel is 2x Heat (converted 31.9, unconverted 36.1) N=4.

[0630] There is a gain of about 1 Ct in the converted ACTB signal when going from 1x heat to 2x heat. This suggests almost all of the DNA is converted. This is supported by the fact that there is also a loss of about 2 Ct's in the unconverted HMBS signal. A 1 Ct increase is logical since we went from heating 50/100 µL to 100/100 µL of DNA-bisulfite sample.

Example 8

Comparison of a DNA Methylation Cartridge With Tube-Based Commercial Kits

[0631] Figure 23A shows a comparison of the user steps required when performing a methylation analysis using cartridge as described herein (left) as compared to the steps required when using commercial kits (QIAamp MinElute Virus Spin Kit (Qiagen, Inc.), and EZ DNA Methylation-Lightning™ Kit (Zymo Research, Inc.)) to perform the same analysis. As can readily be seen the cartridge-based methylation analysis requires far few user steps with a labor time of about 5 minutes as compared to the 2-3 hour labor time required using the kits.

[0632] To compare the results produced by the different methods, 200 µL of serum was purified using the Qiagen kit. The DNA was converted using the Zymo kit, purified with a second spin column and eluted with 10 µL. Ran all 10 µL using converted unmethylated ACTB primers and probes (TSR). In comparison, 200 µL of serum were run in the methylation cartridge as described herein. Results are shown in Figure 23B. As is readily evident, the cartridge method produced results extremely comparable to those obtained using the commercial kits. However, this was accomplished with far less labor and time.

Example 9

Use of DABSO for DNA Conversion

[0633] It was initially attempted to dissolve 5 g DABSO in 5 mL H2O. Ultimately a few mLs of 10M KOH and a mL of water were added and heated to solubilize the DABSO and to raise the pH up to between about pH 5 and pH 5.5 at an estimated final DABSO concentration of ~2.5M.

[0634] Figure 24 shows graphs of tubefills of 750 ng of DNA converted using DABSO or the Zymo conversion reagent. The materials were offboard heated (1μg) in a thermocycler and purified with spin columns and run as tubefills. The 3 different experiments were:

1) 120 μL DABSO/30 μL DNA;

2) 120 μL Zymo/30 μL DNA; and

3) 70 μL Zymo/30 μL DNA (ratio currently in the cartridge).

[0635] As shown in Figure 24, DABSO provided good conversions almost comparable to those obtained using the Zymo reagent.

Example 10

Sensitivity of Detection of Methylated DNA

[0636] To evaluate the sensitivity of detection of DNA methylation, converted *ACTB* gene promoter was detected as a function of copy number using a cartridge as described herein. The goal was to detect less than 25 copies of converted, unmethylated DNA. As previously shown, fallouts were observed at about 10-50 copies (1 fallout each). Similar sensitivity was observed for methylated DNA targets in a serum background.

[0637] Figure 25, panel A, illustrates the detection of methylated DNA in a dilution series (*MGMT* (O-6-Methylguanine-DNA Methyltransferase gene)). As shown therein MGMT was detected down to a level of 78 pg.

[0638] The detection of methylated breast cancer markers *RASSF1A* and *AKR1B1* in MBA-453 cells is shown in Figure 25, panel B. As shown therein, breast cancer markers were detected down to 100 cells.

[0639] The detection of methylated pancreatic cancer markers *ACTB, BNC1,* and *ADAMTS1* in a dilution series is shown in Figure 25, panel C. As shown therein, pancreatic markers were detected down to 25 copies.

[0640] Table 13 shows the hit rate of pancreatic cancer markers *BNC1* and *ADAMTS1* as a function of concentration. As shown therein these markers could be detected below 120 pg. Note a positive "hit rate" is an amplification in either gene for a replicate.

Table 13 illustrates the hit rate for pancreatic marker detection as a function of concentration.

Concentration	Hit rate (#/replicates)
0 pg	0/3
16 pg	6/8
30pg	5/8
60 pg	4/8
120 pg	4/4

Example 11

Reverse Complement Multiplex assay for Both Strands

[0641] Figure 26 illustrates the results for a reverse complement multiplex assay for both DNA strands. Following bisulfite conversion, both strands lose their complementarity. Thus, primer and probe sets have to be designed for one strand or the other, and result in unique amplicons. In addition to providing "more opportunities", this approach might potentially help with sensitivity (at LOD, if only one strand or the other ends up in the tube, this approach would ensure the signal gets picked up).

[0642] The multiplex assay allows detection of different CpGs at the same promoter site. The reverse complement multiplex provides more queries on target and the possibility to pick up heterogamous methylation.

Example 12

Detection of DNA Methylation and Mutation in a Single Cartridge.

[0643] In certain embodiments the multiplex PCR reactions can contain primers and probes that permit the detection of mutations in addition to methylation in the same cartridge. Figures 27A illustrates the detection of methylated *BNC1* and *ADAMTS1* along with the KRAS G12D mutation along with control BG (Top Panel) and the detection of methylated *BNC1* and *ADAMTS1* along with the *KRAS* wildtype along with control BG (Bottom Panel).

[0644] Figure 27B illustrates the simultaneous detection of *BNC1* and *ADAMTS1* methylation in PANC-1 cells (top panel) and MIA-PaCa cells (bottom panel) along with the *KRAS* G12D mutation.

Example 13

Multiplex Optimization of Pancreatic Cancer.

[0645] It was determined that methylation analysis of *ADAMTS1*, *BNC1*, (and certain other genes) permits detection and/or staging of pancreatic cancer. Accordingly, the initial multiplex assay for *BNC1* and *ADAMTS1* was optimized to facilitate incorporation of probes for other genes. To optimize this assay temperature gradients were run on external and internal PCRs for forward/reverse bisulfite converted strands. Single-plexes (fwd/rev for each gene) were run at external temperatures of 56°C, 58°C, and 60°C and internal temperatures of 64 °C, 66 °C, and 68 °C (*see, e.g.*, Figure 28). In certain embodiments the assays were developed as two 4-plexes for *BNC1* and *ADAMTS1* and two other genes, one 4-plex for methylation analysis of a forward strand and one 4-plex for methylation analysis of a reverse strand.

[0646] The probes were combined into two sets (*see,* Figure 29) based on preferred reaction conditions (salt conditions 40 mM (LS), 60 mM (MS), 80 mM (HS) KCl, 15 mM NH_4SO_4) and optimized for specificity. The final optimized salt condition for multiplex 1 was 80mM KCl, 5mM $MgCl_2$, 20mM Tris pH 8.5, and 10mM NH_4 and for multiplex 2 was 62mM KCl, 4mM $MgCl_2$, 20mM Tris pH 8.5, and 10mM NH_4.

Example 14

Detection of *MGMT* Methylation

[0647] The O(6)-methylguanine-DNA methyltransferase (*MGMT*) gene encodes a DNA repair enzyme that can abrogate the effects of alkylating chemotherapy such as temozolamide. If the *MGMT* gene is active, the damage is rapidly repaired. It is believed that malignant gliomas may have the *MGMT* gene inactivated due to methylation of its promoter region. Methylated *MGMT* gene is a predictive indicator for better response to chemotherapy (as the tumor has no means to repair the DNA damage induced by the alkylating agent).

[0648] Primers and probes were developed for the detection of *MGMT* methylation as illustrated in Figure 30 and summarized below in Table 14. In particular, Figure 30 illustrates the converted template with CPGs (as determined from pyrosequencing) shown in grey. As illustrated after bisulfite conversion the forward and reverse strand are no longer complementary permitting separate analysis of each strand.

Table 14. Illustrative primer/probe set for detection of *MGMT* methylation (*see, e.g.,* Figure 30).

Probe		Probe Type	Sequence	SEQ ID NO
External	22422	MGMT Fwd-4	GTTTT(T*)AGAAYG(T*)TTTGYGTTT	263
	22423	MGMT Rev-4	AAAAAAC(T*)CCRCACTCTTCC	265
Internal	22150	MGMT Fwd-2	TTTCGACGTTCGTAGGTTTTCGC	266
	22151	MGMT Rev-2	GCACTCTTCCGAAAACGAAACG	267
	22419	MGMT TaqMan-2	Fluor-CCAAACAC(T*)CACCAAATC(N*)CAAAC	268

[0649] To evaluate detection sensitivity a *MGMT* dilution series (5 ng to 78 pg *MGMT* DNA in a background of 20 ng of HS DNA)) was evaluated using ACTB as a control. In an illustrative experiment, 78pg of methylated *MGMT* DNA was only about 10 cycles off the Ct of only unmethylated HS DNA.

[0650] As shown in Figure 31 results produced using the methylation cartridge described herein for the detection of *MGMT* methylation was compared to the results produced by pyrosequencing for extracted DNA (Fig. 31, top) and for a FFPE sample (Fig. 31, bottom). Pyrosequencing typically uses a cutoff between 10-15% to determine patient stratification. We used an arbitrary cutoff of 12.5 (between *ACTB* and *MGMT*) to match pyrosequencing results as closely as possible. Accordingly, in this example a cutoff was set at delta Ct=12.5 and calculated concordance with >15% methylation. The cartridge analysis of the extracted DNA show a sensitivity of 90% and a specificity at 86% while the cartridge analysis of the FFPE sample showed a sensitivity of 88% and a specificity of 95%.

[0651] It is noted that specificity can be improved in two ways: 1) the annealing temperature can be increased as the 62°C annealing temperature was rather low. Additionally methylation probes that cover 3 (or more) CpGs can be utilized.

Example 15

Detection of *BRCA1* Methylation

[0652] BRCA1 is a caretaker gene responsible for repairing DNA. It is believe that *BRCA1* is involved in homologous, recombination, non-homologous end joining, and nucleotide excision repair. Women with an abnormal *BRCA1* gene have an 80% chance of developing breast cancer.

[0653] Without being bound to a particular theory, it is believed that *BRCA1* methylation is a potential predictive marker of response to chemotherapy in triple negative BC patients. Study of NSCLC patient's treated with cisplatin showed those with low *BRCA1* expression had improved survival rates. High levels reduced the effectiveness of chemotherapy by repairing the damage caused to cancer cells.

[0654] In view of these, and other, observations cartridges and methods of use were developed for detection of BRCA1 methylation. In particular, the PCR condition were optimized as follows: 1) External temperature was evaluated between 56-62°C and we settled on a 3 step 56ºC annealing PCR protocol; 2) Internal temperature was evaluated between 64ºC-70 °C and we settled on a two-step 68ºC annealing PCR protocol. Results are shown in Figure 32.

[0655] For *BRCA1*, a one target assay was tested with the *ACTB* control gene. Eight different cell lines were tested and the effect of adding NH_4 was compared (*see,* Figure 33). *BRCA1* methylation was expected to be observed in the 3199 cell line.

Example 16

Detection of Gene Methylation Associated With Lung Cancer.

[0656] A three target methylation assay for genes whose methylation is associated with lung cancer (*SOX17, CD01, TAC1*) was tested along with the *ACTB* control gene. The data shown in Figure 34 indicate that, as expected, the 3 targets do not come up in a background of normal plasma but are present to some degree in three different lung cancer cell lines.

[0657] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

2016277943 04 Aug 2022

CLAIMS

1. A method of producing a converted nucleic acid for determining its methylation state, said method comprising:

i) contacting a biological sample comprising a nucleic acid to a first matrix material comprising a first column or filter where said matrix material binds and/or filters nucleic acids in said sample and thereby purifies the DNA;

ii) eluting any bound DNA from the first matrix material and denaturing the eluted or purified DNA to produce denatured DNA;

iii) heating the denatured DNA in the presence of a bisulfite reagent to produce a deaminated nucleic acid;

iv) contacting said deaminated nucleic acid to a second matrix material comprising a second column to bind said deaminated nucleic acid to said second matrix material;

v) desulfonating the bound deaminated nucleic acid and/or simultaneously eluting and desulfonating the bound deaminated nucleic acid by contacting the bound deaminated nucleic acid with an alkaline solution to produce a converted nucleic acid;

vi) if not previously eluted, eluting said converted nucleic acid from said second matrix material; and

vii) amplifying the converted nucleic acid, wherein at least steps iv) through vi) are performed in a single reaction cartridge;

wherein said cartridge comprises a column comprising said first matrix material, a sample receiving chamber, a reaction channel or chamber that can be temperature controlled and subjected to thermocycling, a plurality of chambers for containing reagents and/or buffers, and wherein when in use at least one of said chambers contains a desulfonation and/or elution buffer;

wherein said sample receiving chamber, said columns, said plurality of chambers, and said reaction channel or chamber are selectively in fluid communication by microfluidic channels and valves;

wherein:

said sample receiving chamber, said columns, said plurality of chambers, said reaction channel or chamber or a port into said reaction channel or chamber, are disposed around a central valve and selectively in fluid communication with a channel in said central valve, wherein said central valve is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with said central valve; and optionally

said amplification is carried out by polymerase chain reaction (PCR), wherein the heating with the bisulfite reagent is performed in the reaction channel or chamber that is later used for PCR, optionally wherein:

at least steps iii) through vi) are performed in a single reaction cartridge; or

at least steps ii) through vi) are performed in a single reaction cartridge; or

at least steps i) through vi) are performed in a single reaction cartridge.

2. The method according to claim 1, wherein step vii) is performed in the same reaction cartridge.

3. The method according to any one of claims 1-2, wherein methylation specific PCR is performed in said cartridge.

4. The method according to any one of claims 1-3, wherein nucleic acid sequencing is performed in said cartridge or in a device coupled to said cartridge.

5. The method according to any one of claims 1-4, wherein, when in use, at least one of said chambers contains a reagent that provides bisulfite ions.

6. The method according to any one of claims 1-5, wherein said cartridge, when in use, comprises:

a first chamber containing a sample;

a second chamber containing a guanidinium thiocyanate-ethanol (GTC-EtOH) solution;

a third chamber containing a bisulfite reagent;

a fourth chamber containing a buffer;

a fifth chamber containing a rinse solution; and

a sixth chamber containing an elution/desulfonation reagent, optionally wherein said first chamber contains said sample in a GTC-EtOH-Tween extraction/ precipitation reagent.

7. The method according to claim 6, wherein:

the GTC-ETOH-Tween buffer is provided as a component of the cartridge; and/or

the bisulfite reagent is provided as a component of the cartridge.

8 The method according to any one of claims 1-7, wherein said cartridge comprises a seventh chamber containing PCR primers and/or probes and/or PCR enzymes.

9. The method of claim 8, wherein said PCR primers, and/or probes, and/or enzymes are provided as beads.

10. The method according to any one of claims 1-9, wherein said bisulfite ion is provided as a compound selected from the group consisting of sodium metabisulfite, potassium bisulfite, cesium bisulfite, ammonium bisulfite, and DABSO.

11. The method according to any one of claims 1-10, wherein:

said eluting the bound DNA comprises eluting and denaturing said DNA with an alkaline solution with a pH greater than about pH 10.5 or greater than about pH 12; and/or

said incubating the eluted DNA with bisulfite ions to produce a deaminated nucleic acid comprises transferring the DNA in a concentrated bisulfite solution into the reaction channel or chamber in said cartridge and heating said mixture; and/or

said contacting said deaminated nucleic acid to a second matrix material comprises mixing the DNA-bisulfite solution with GTC-EtOH and dispensing the solution over said second matrix material; and/or

said desulfonating the bound deaminated nucleic acid comprises eluting the DNA from said second column with a high pH desulphonation buffer and incubating said solution.

12. The method according to any one of claims 1-11, wherein after the incubation with bisulfite ions, said reaction channel or chamber is washed with a buffer to remove the residual bisulfite and neutralize pH.

13. The method of claim 12, wherein said reaction channel or chamber is sufficiently free of bisulfite ion to permit PCR to be performed in said channel or chamber.

14. The method according to any one of claims 1-13, wherein methylation specific PCR is performed to determine methylation of target nucleic acid sequences.

15. The method according to any one of claims 1-14, wherein a bisulfite reaction and a PCR reaction, or a desulfonation reaction and a PCR reaction, or a bisulfite reaction, a desulfonation reaction and a PCR reaction are performed in the same reaction tube or chamber.

16. A cartridge for determining the methylation state of a nucleic acid, said cartridge comprising:

a column comprising a first matrix material, a sample receiving chamber, a reaction channel or chamber, a plurality of chambers for containing reagents and/or buffers, and when in use at least one of said chambers contains a bisulfite reagent, and when in use at least one of said chambers contains a desulphonation/elution buffer, and wherein said cartridge optionally comprises a second column comprising a second matrix material;

wherein said sample receiving chamber, said columns, said plurality of chambers, and said reaction channel or chamber are selectively in fluid communication by microfluidic channels and valves;

wherein:

said sample receiving chamber, said column(s), said plurality of chambers, said reaction channel or chamber or a port into said reaction channel or chamber, are disposed around a central valve and selectively in fluid communication with a channel in said central valve, wherein said central valve is configured to accommodate a plunger that is capable of drawing fluid into or out of a chamber in fluid communication with said central valve; and optionally

said cartridge is capable of performing bisulfite conversion in the reaction channel or chamber, which is later used for polymerase chain reaction (PCR).

17. The cartridge according to claim 16, wherein said cartridge is capable of performing bisulfite conversion in the reaction channel or chamber, which is later used for polymerase chain reaction (PCR).

18. The cartridge according to any one of claim 16 or 17, wherein said bisulfite reagent comprises a compound selected from the group consisting of ammonium bisulfite sodium metabisulfite, potassium bisulfite, cesium bisulfite, and DABSO.

2016277943 04 Aug 2022

19. The cartridge according to any one of claims 16-18, wherein said cartridge comprises one or more chambers containing one or more reagents selected from the group consisting of methylation specific PCR primers, methylation specific PCR probes, PCR enzyme(s), and PCR reaction buffer.

20. The cartridge according to claim 19, wherein said PCR primers, and/or probes, and/or enzymes are provided as beads.

21. The cartridge according to any one of claims 16-20, wherein the bisulfite reagent is provided as a component of the cartridge.

22. A system for determining the methylation of a nucleic acid in a biological sample, said system comprising:

an enclosure configured to contain one or more sample processing modules, each sample processing module configured to hold a removable cartridge according to any one of claims 16-21;

where said system is configured to operate the sample processing modules to perform sample processing to determine methylation of one or more target nucleic acids and optionally to determine the level of one or more target DNA sequences within a corresponding removable sample cartridge, wherein said processing on a sample within the corresponding removable sample cartridge performs a method according to any one of claims 1-15.

23. The system of claim 22, wherein:

said system is configured to contain one sample processing module; or

said system is configured to contain at least two sample processing modules, or at least 4 sample processing modules, or at least 8 sample processing modules, or at least 12 sample processing modules, or at least 16 sample processing modules, or at least 20 sample processing modules, or at least 24 sample processing modules, or at least 28 sample processing modules, or at least 32 sample processing modules, or at least 64 sample processing modules, or at least 128 sample processing modules.

24. The system according to any one of claims 22-23, wherein:

said modules comprise one or more heating plates to heat a reaction chamber or channel in said cartridge; and/or said modules comprise a fan configured to cool the reaction channel or chamber in said cartridge; and/or

said modules comprise circuitry to pass information (*e.g.*, optical information) to a computer for analysis; and/or

said modules comprise optical blocks to provide excitation and/or detection of one or more optical signals produced by reactions in said cartridge.

25. The system according to any one of claims 22-24, wherein:

said system is configured to operate said cartridge to perform a method according to any one of claims 1-15; and/or

said system is configured to operate said cartridge to:

bind a sample to a column;

elute DNA from the column and combine said DNA with a conversion reagent;

heat the DNA/conversion reagent solution in a reaction chamber or tube to produce converted DNA;

bind the converted DNA to a column;

desulphonate and elute the DNA from the column; and

perform PCR on the eluted desulphonated DNA in a reaction chamber or tube.

26. The system of claim 25, wherein said PCR is performed in the same reaction chamber or tube where the DNA/conversion reagent solution was previously heated.

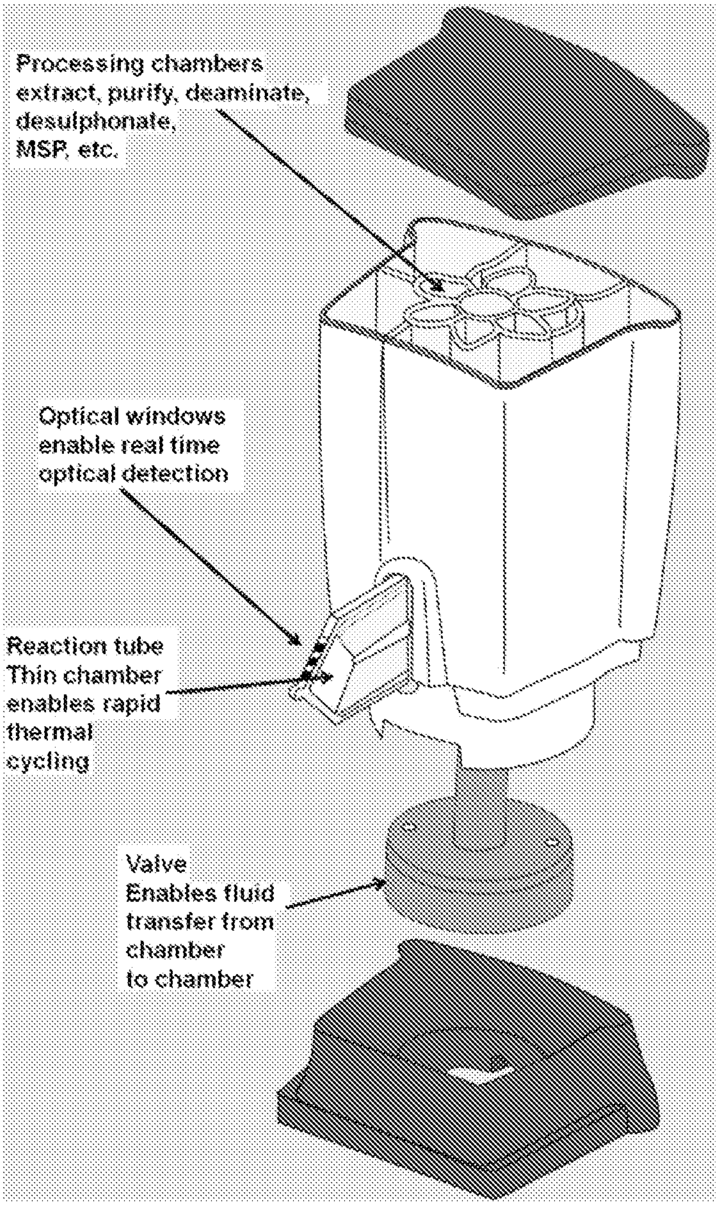

Processing chambers
extract, purify, deaminate,
desulphonate,
MSP, etc.
Optical windows
enable real time
optical detection
Reaction tube
Thin chamber
enables rapid
thermal
cycling
Valve
Enables fluid
transfer from
chamber
to chamber

Fig. 1A

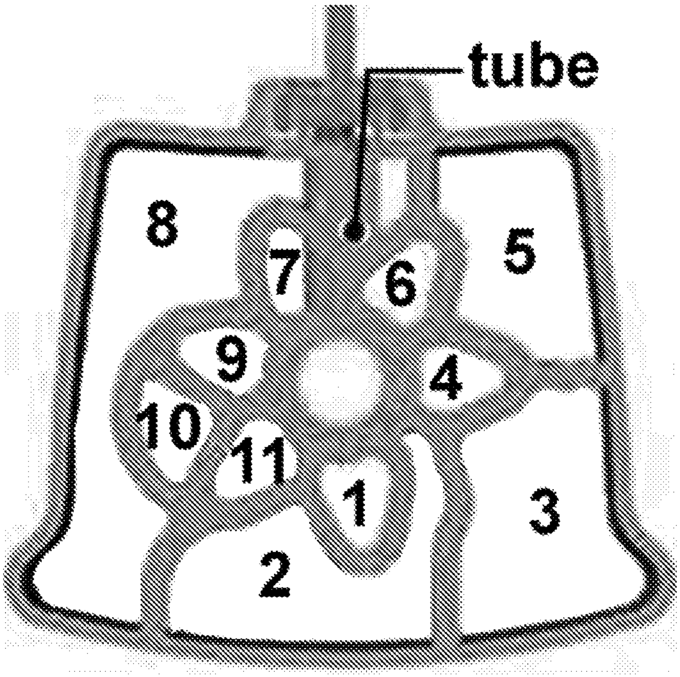
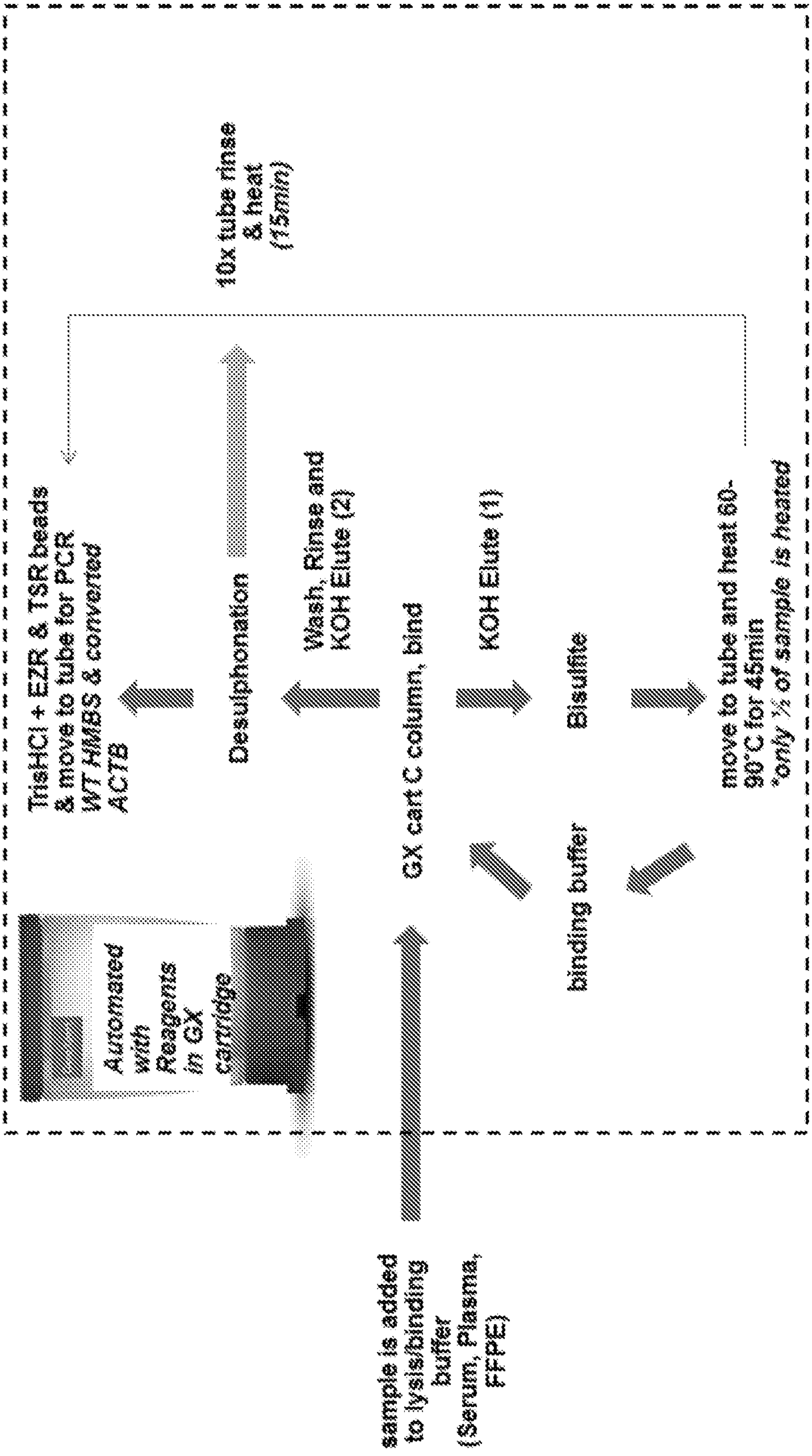


Fig. 1B



Automated with Reagents in GX cartridge
sample is added to lysis/binding buffer (Serum, Plasma, FFPE)
GX cart C column, bind
binding buffer
KOH Elute (1)
Bisulfite
move to tube and heat 60-90°C for 45min
*only ½ of sample is heated
Wash, Rinse and KOH Elute (2)
Desulphonation
TrisHCl + EZR & TSR beads & move to tube for PCR
WT HMBS & converted ACTB
10x tube rinse & heat
(15min)

Fig. 1C

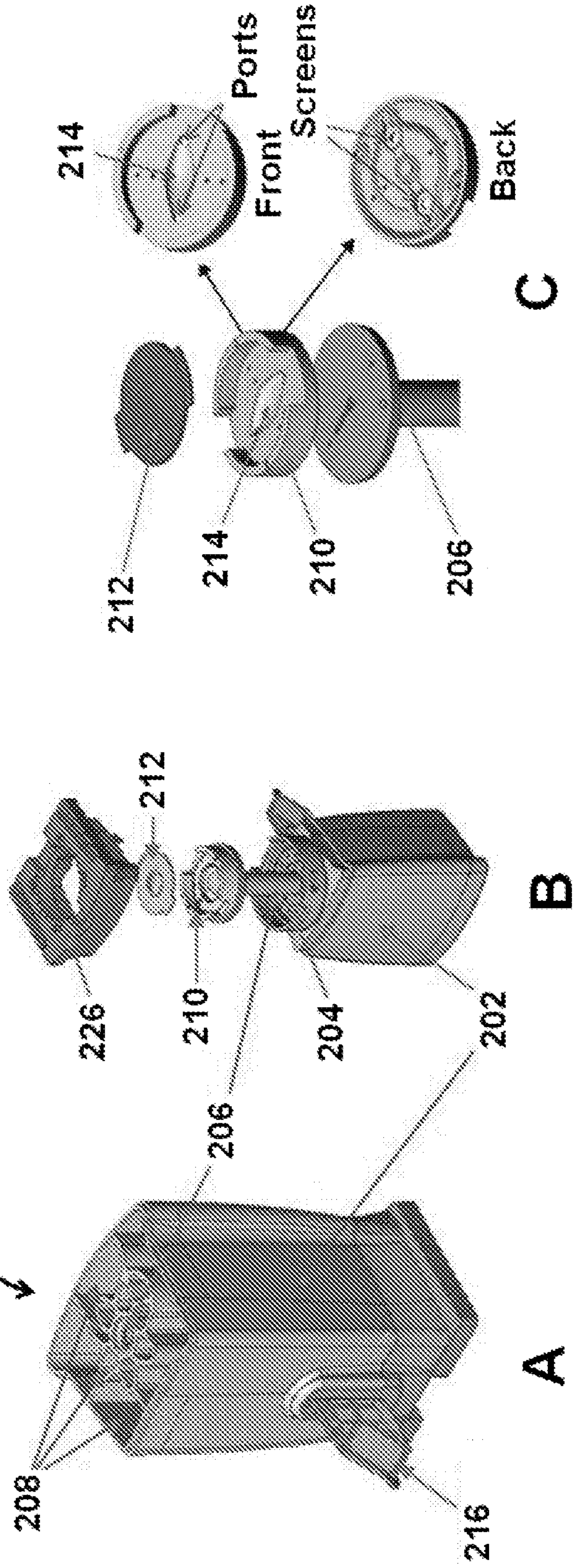

214
Ports
Front
Screens
Back
C
212
214
210
206
226
210
204
202
212
B
200
206
202
208
216
A

Fig. 2

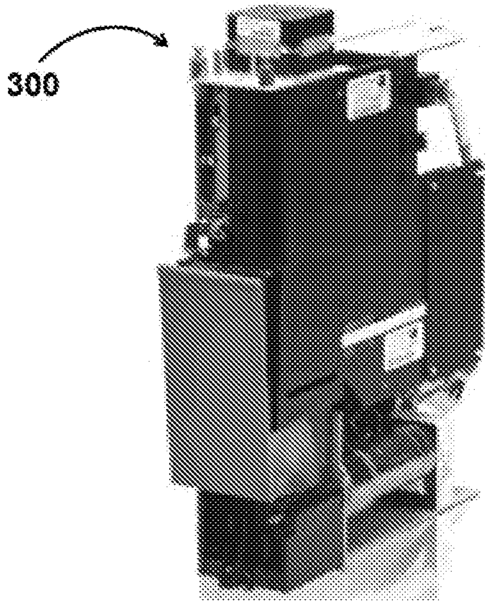


Fig. 3A

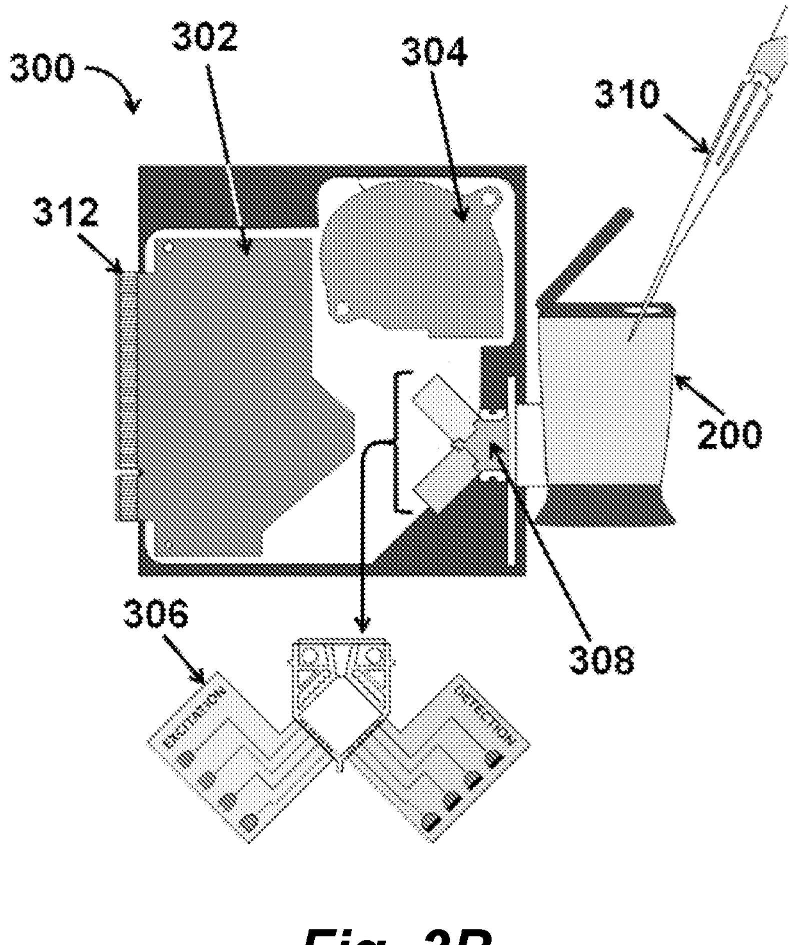


Fig. 3B

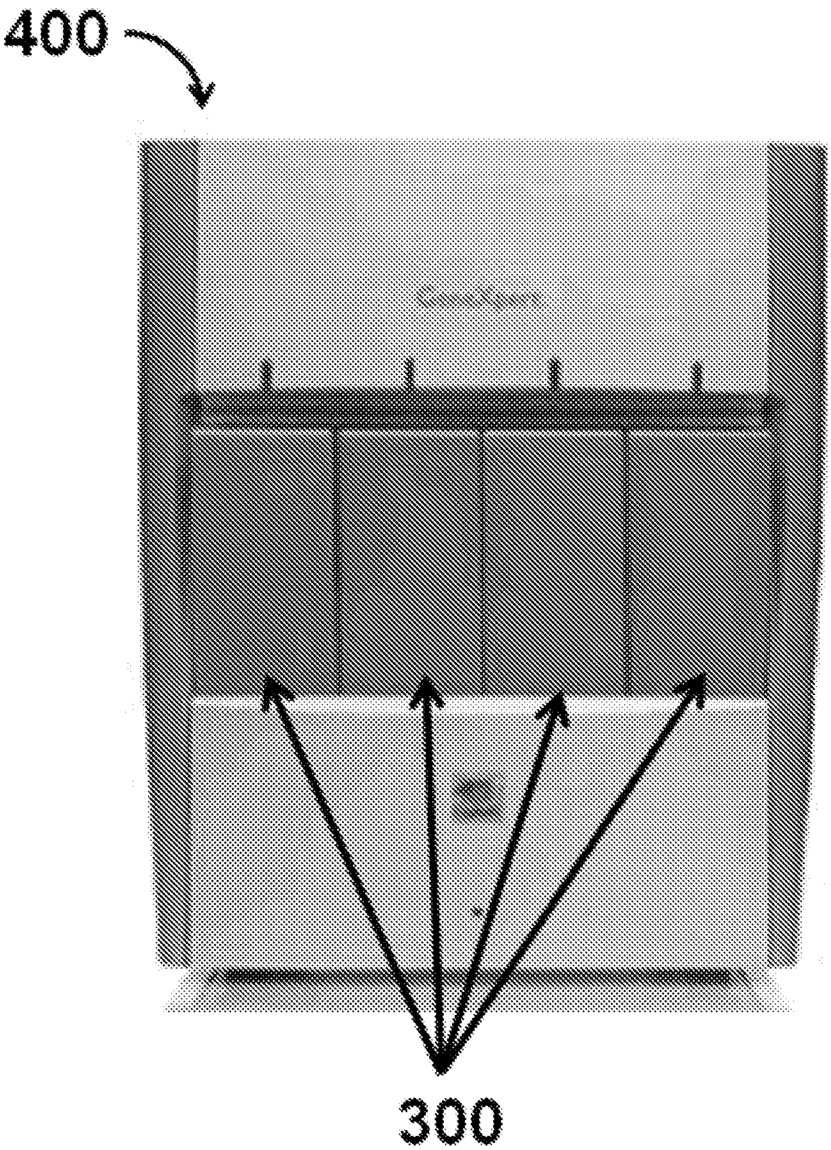


Fig. 3C

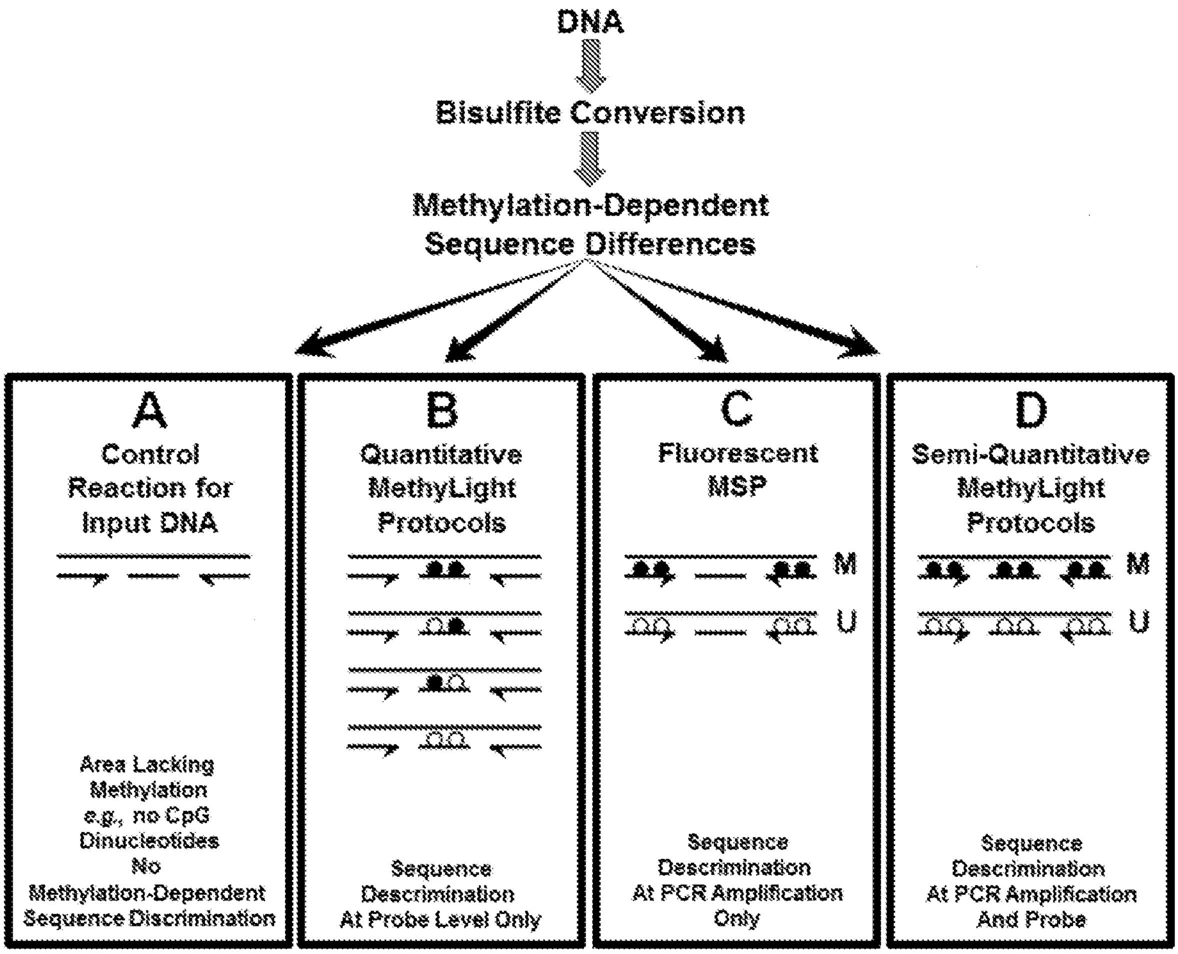

DNA
Bisulfite Conversion
Methylation-Dependent Sequence Differences
A
Control Reaction for Input DNA
Area Lacking Methylation e.g., no CpG Dinucleotides No Methylation-Dependent Sequence Discrimination
B
Quantitative MethyLight Protocols
Sequence Descrimination At Probe Level Only
C
Fluorescent MSP
M
U
Sequence Descrimination At PCR Amplification Only
D
Semi-Quantitative MethyLight Protocols
M
U
Sequence Descrimination At PCR Amplification And Probe

Fig. 4A


Single probe (MSP) method:
FW
PR3
RV

Fig. 4B

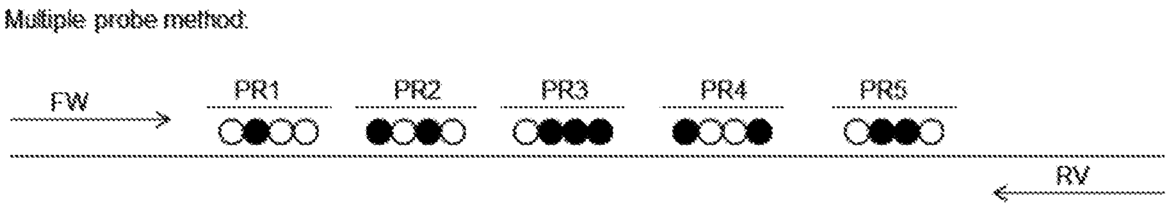

Multiple probe method:
FW
PR1
PR2
PR3
PR4
PR5
RV

Fig. 4C

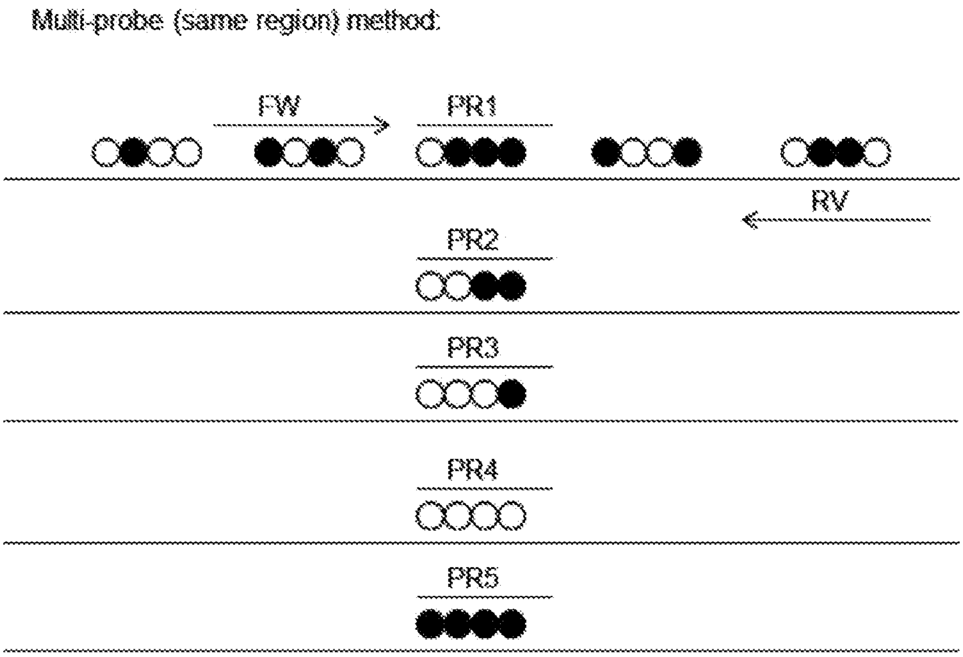
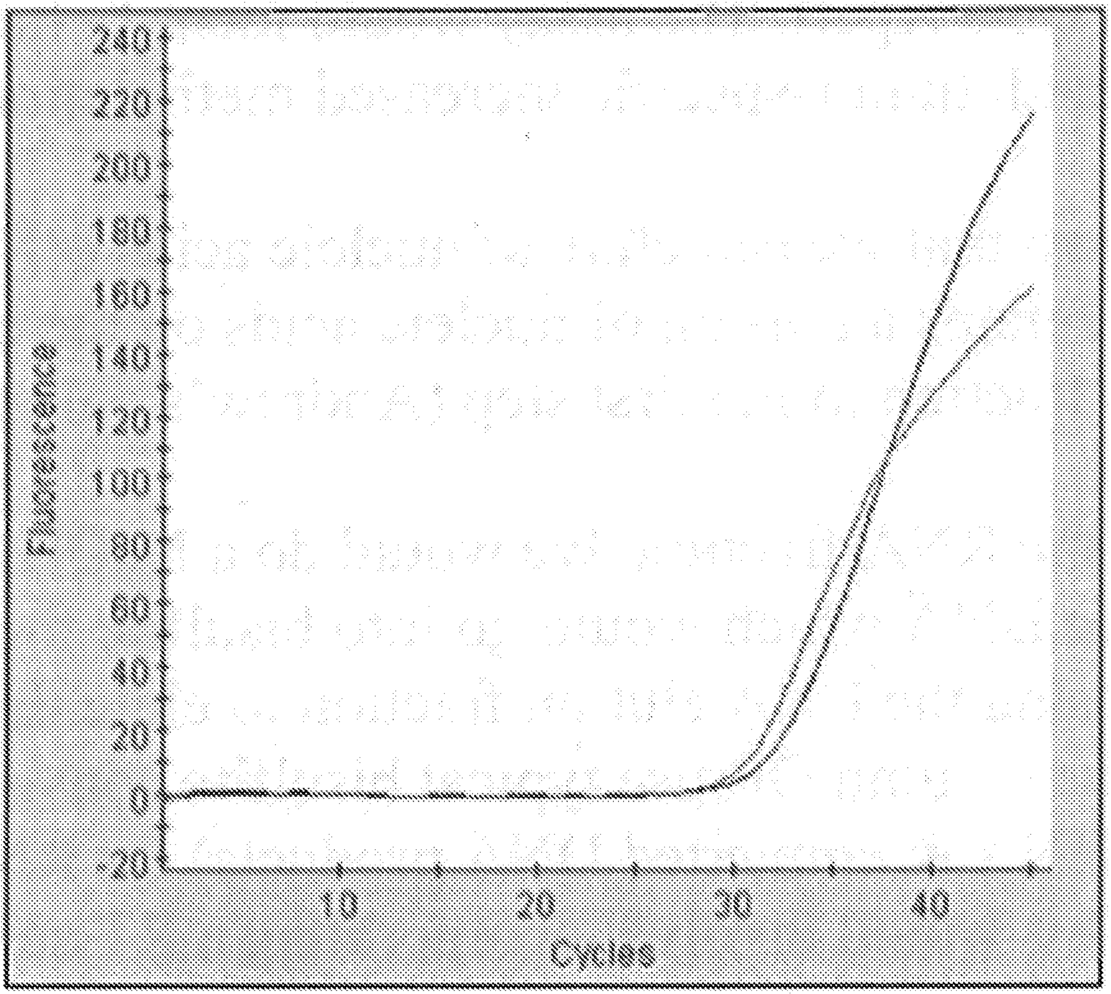

Multi-probe (same region) method:
FW
PR1
RV
PR2
PR3
PR4
PR5

Fig. 4D



Fluorescence
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
10
20
30
40
Cycles

Fig. 5

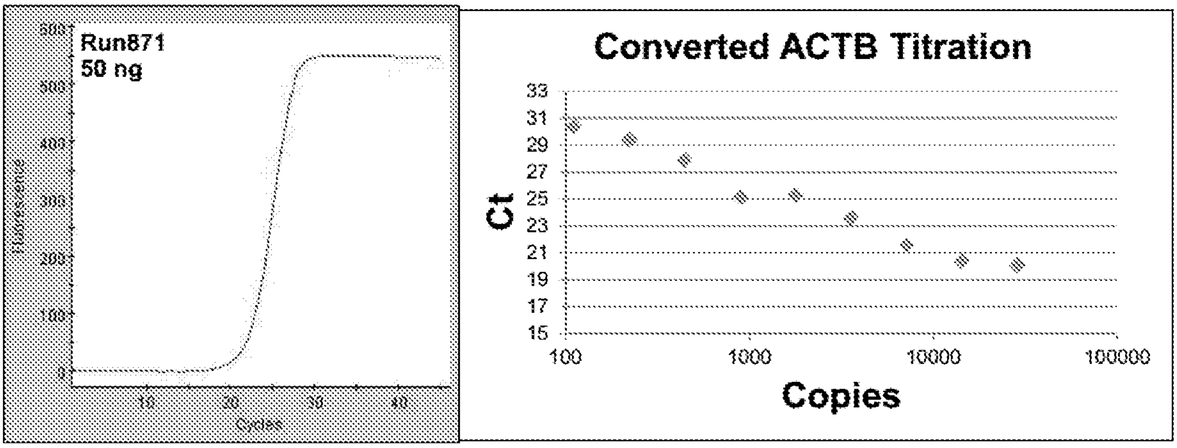

Run871
50 ng
Fluorescence
Cycles
Converted ACTB Titration
Ct
Copies
33
31
29
27
25
23
21
19
17
15
100
1000
10000
100000

Fig. 6A

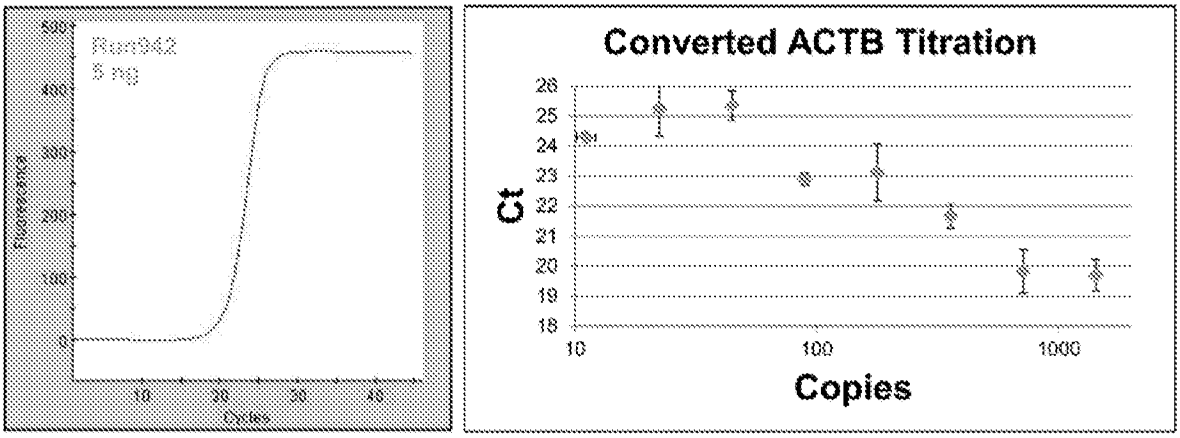

Run942
5 ng
Fluorescence
Cycles
Converted ACTB Titration
Ct
Copies
26
25
24
23
22
21
20
19
18
10
100
1000

Fig. 6B

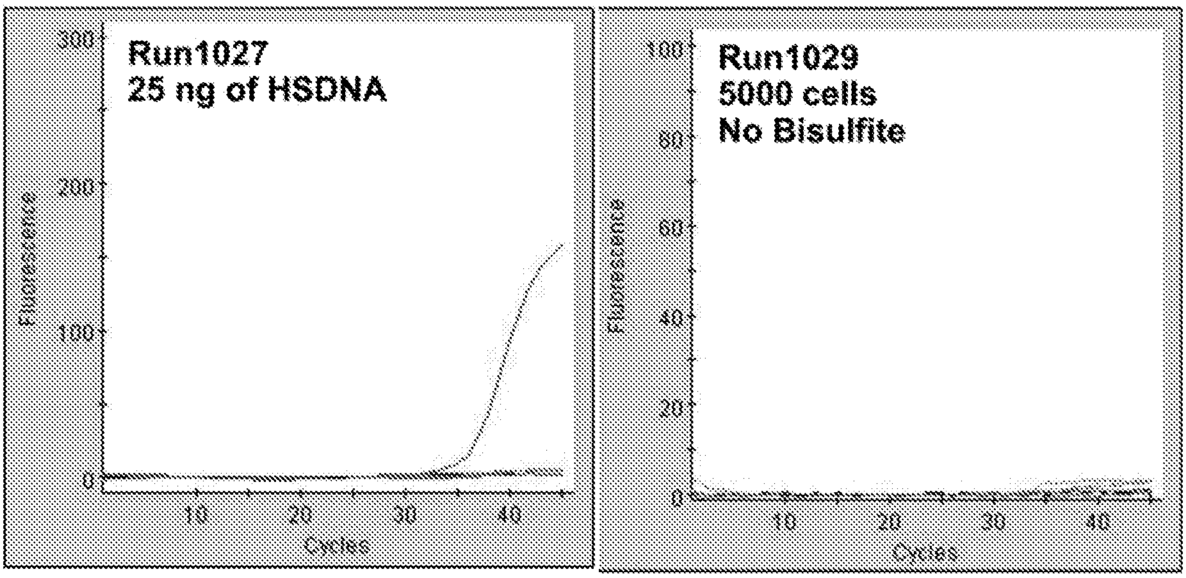

Run1027
25 ng of HSDNA
Fluorescence
Cycles
Run1029
5000 cells
No Bisulfite
Fluorescence
Cycles

Fig. 7A

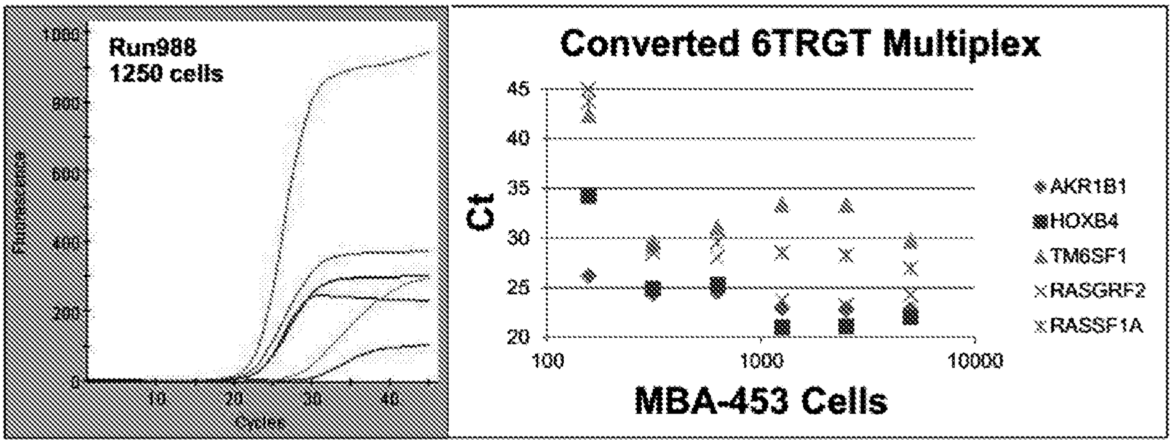

Run988
1250 cells
Cycles
Converted 6TRGT Multiplex
Ct
MBA-453 Cells
AKR1B1
HOXB4
TM6SF1
RASGRF2
RASSF1A

Fig. 7B

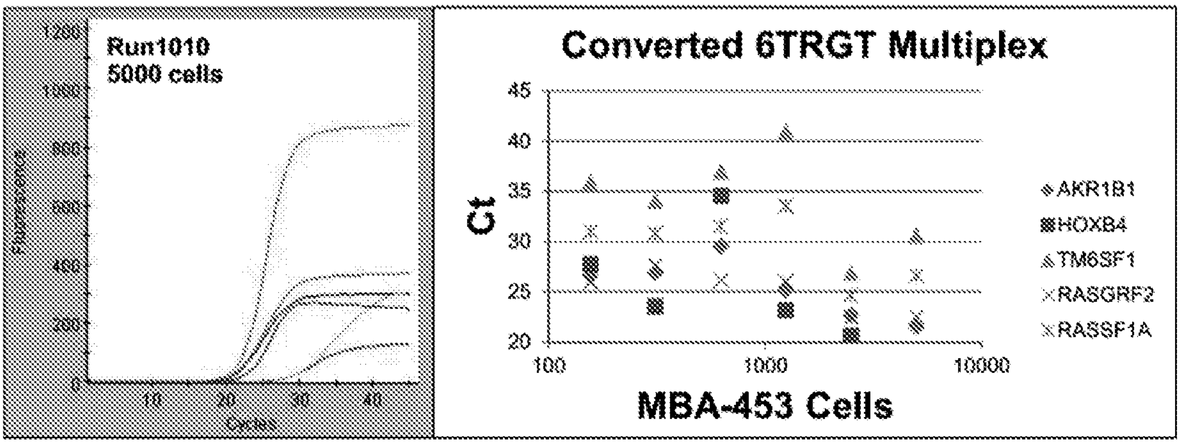

Run1010
5000 cells
Fluorescence
Cycles
Converted 6TRGT Multiplex
Ct
MBA-453 Cells
AKR1B1
HOXB4
TM6SF1
RASGRF2
RASSF1A

Fig. 7C

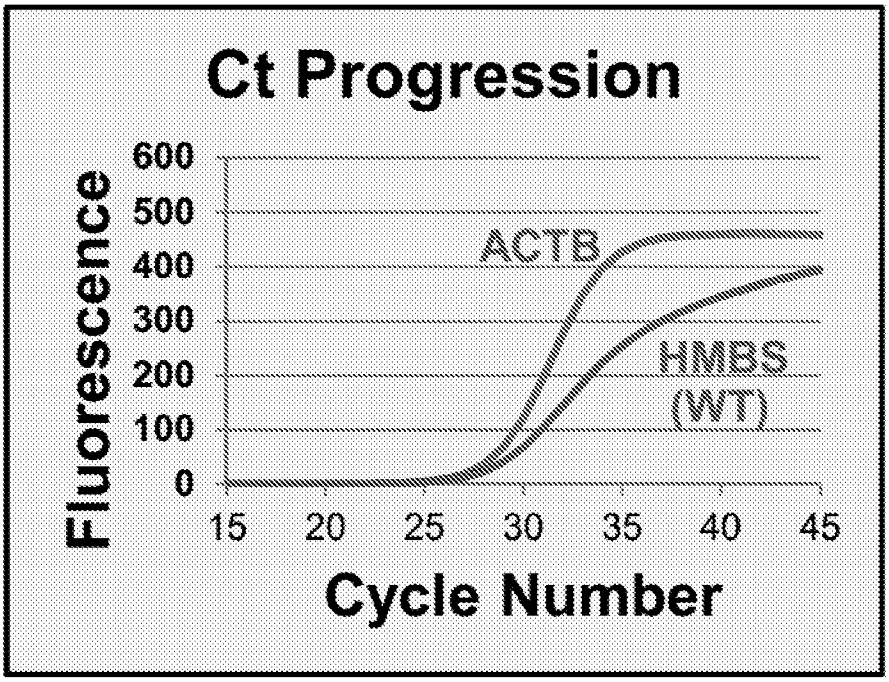

Ct Progression
Fluorescence
Cycle Number
ACTB
HMBS
(WT)

Fig. 8

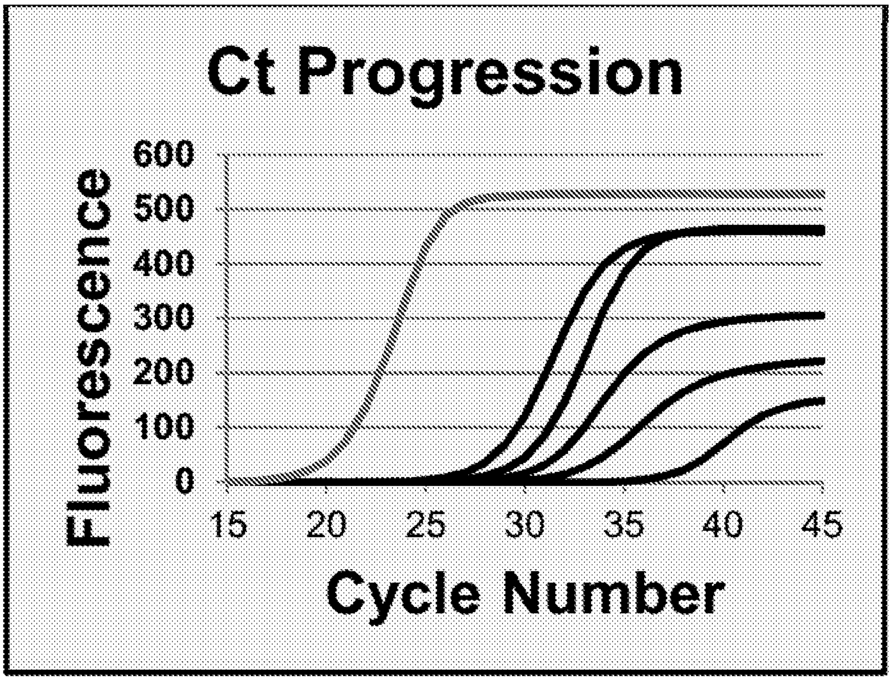

Ct Progression
Fluorescence
600
500
400
300
200
100
0
15
20
25
30
35
40
45
Cycle Number

Fig. 9

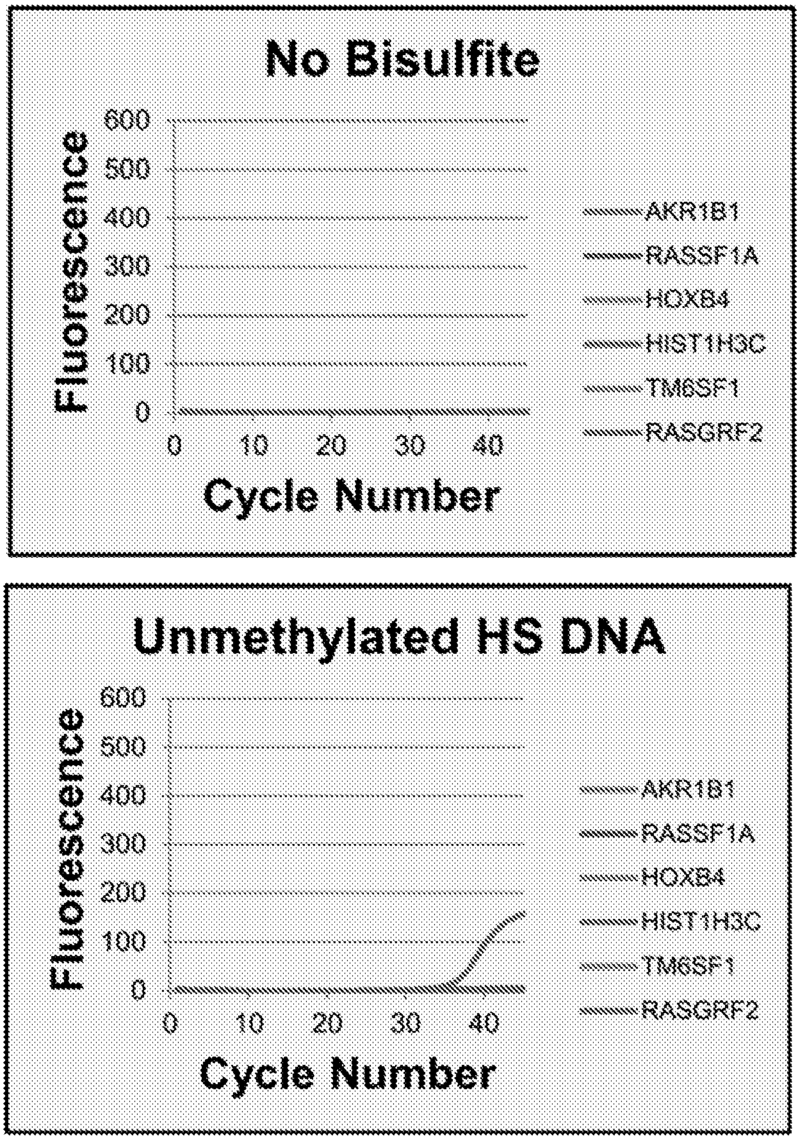
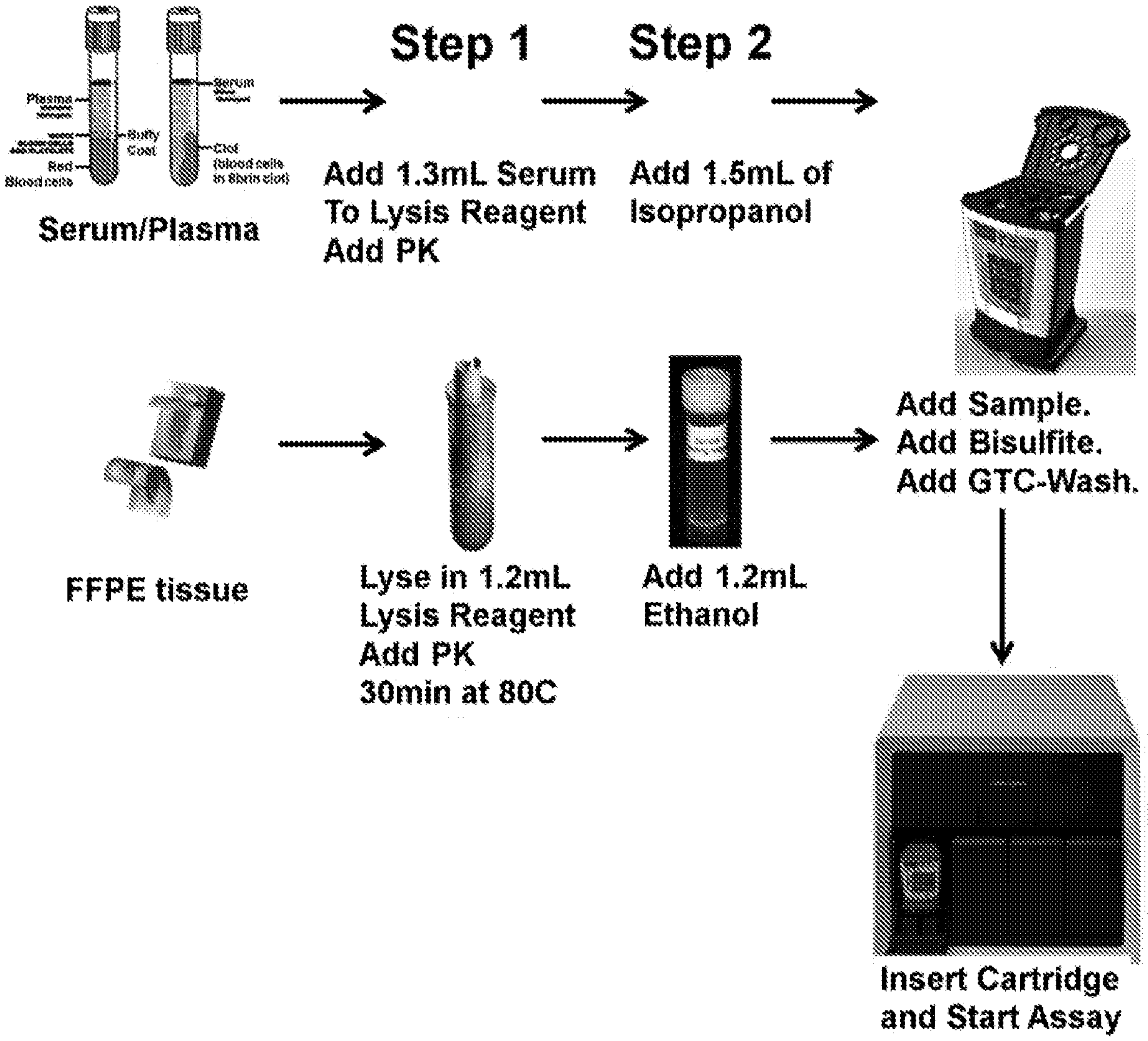

No Bisulfite
Fluorescence
600
500
400
300
200
100
0
0
10
20
30
40
Cycle Number
AKR1B1
RASSF1A
HOXB4
HIST1H3C
TM6SF1
RASGRF2
Unmethylated HS DNA
Fluorescence
600
500
400
300
200
100
0
0
10
20
30
40
Cycle Number
AKR1B1
RASSF1A
HOXB4
HIST1H3C
TM6SF1
RASGRF2

Fig. 10



Step 1
Step 2
Plasma
Buffy Coat
Red Blood cells
Serum
Clot (blood cells in fibrin clot)
Serum/Plasma
Add 1.3mL Serum To Lysis Reagent Add PK
Add 1.5mL of Isopropanol
FFPE tissue
Lyse in 1.2mL Lysis Reagent Add PK 30min at 80C
Add 1.2mL Ethanol
Add Sample.
Add Bisulfite.
Add GTC-Wash.
Insert Cartridge and Start Assay

Fig. 11

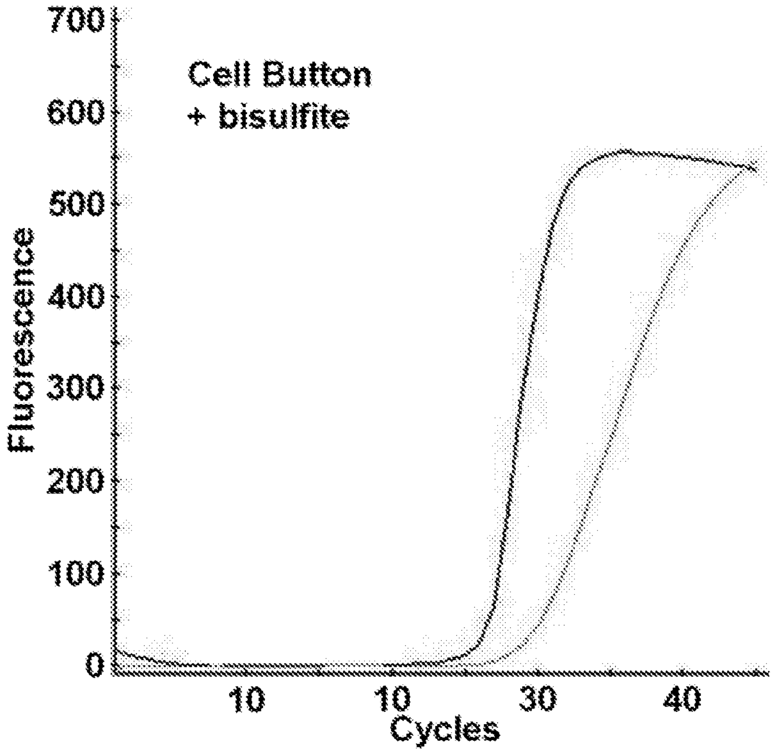
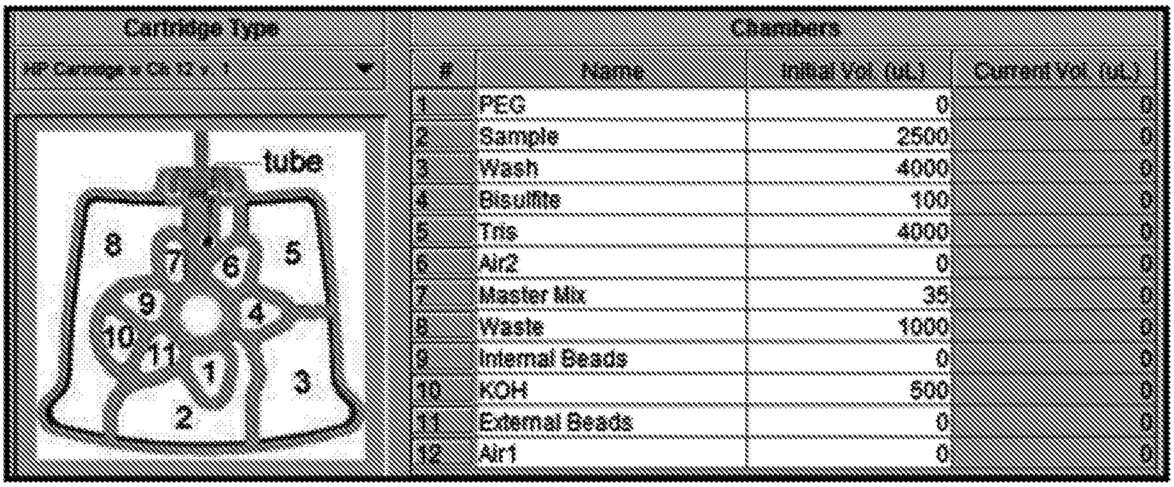

700
600
500
400
300
200
100
0
Fluorescence
Cell Button
+ bisulfite
10
10
30
40
Cycles

Fig. 12



Chambers			
#	Name	Initial Vol. (uL)	Current Vol. (uL)
1	PEG	0	0
2	Sample	2500	0
3	Wash	4000	0
4	Bisulfite	100	0
5	Tris	4000	0
6	Air2	0	0
7	Master Mix	35	0
8	Waste	1000	0
9	Internal Beads	0	0
10	KOH	500	0
11	External Beads	0	0
12	Air1	0	0

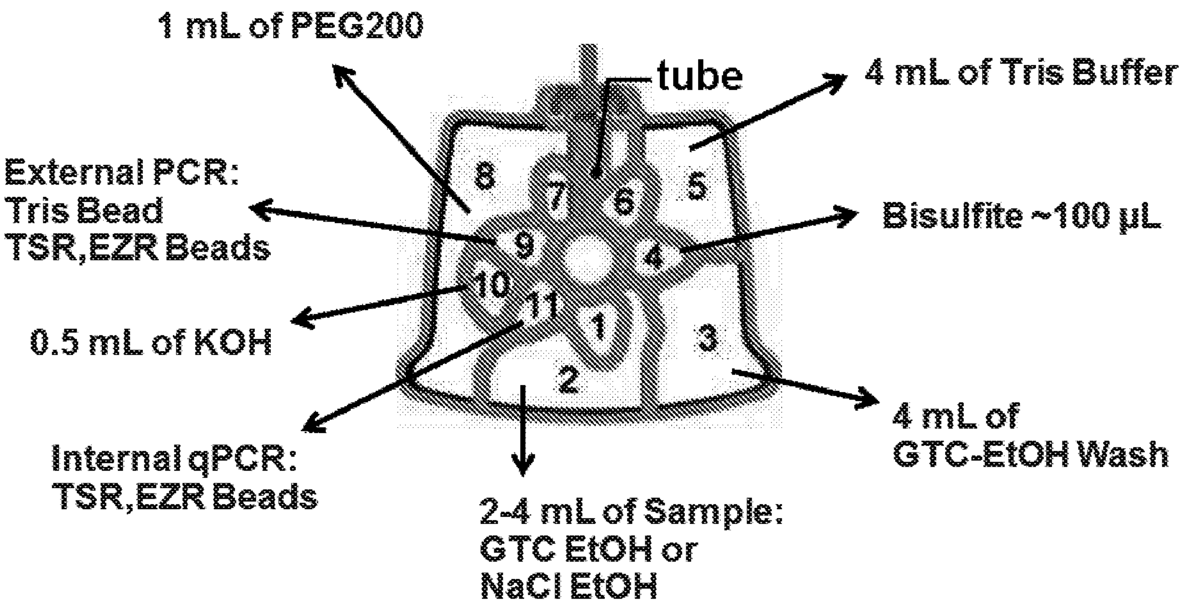


Fig. 13A

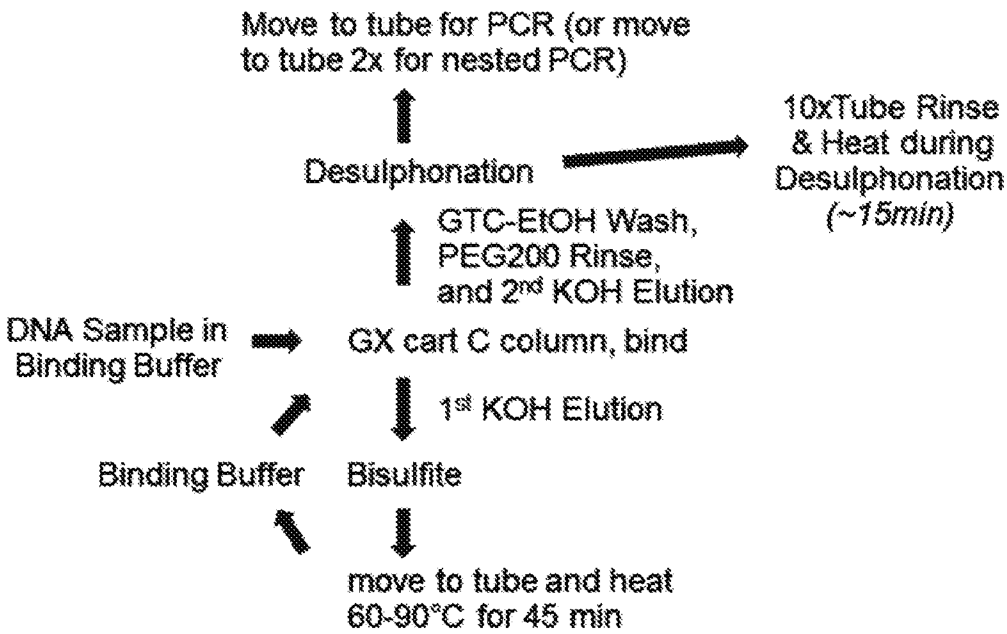


Fig. 13B

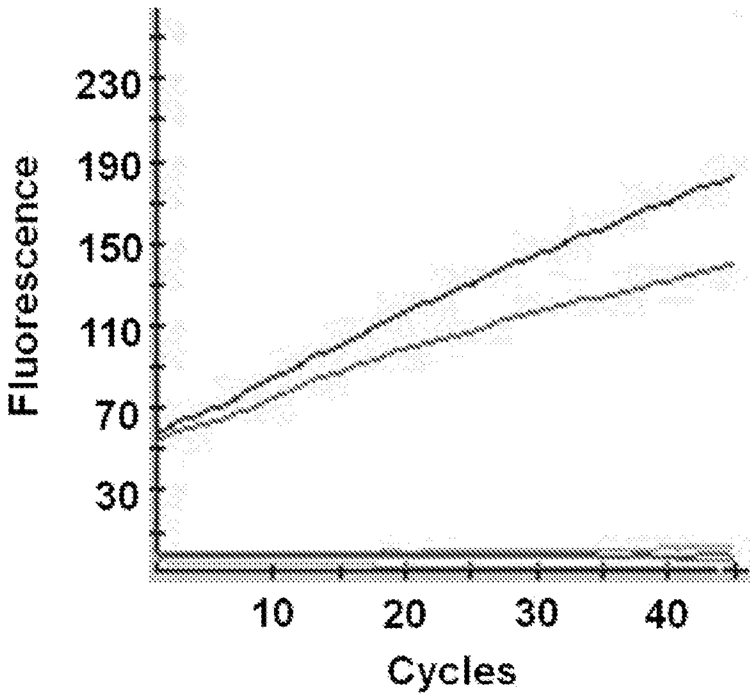


Fig. 14

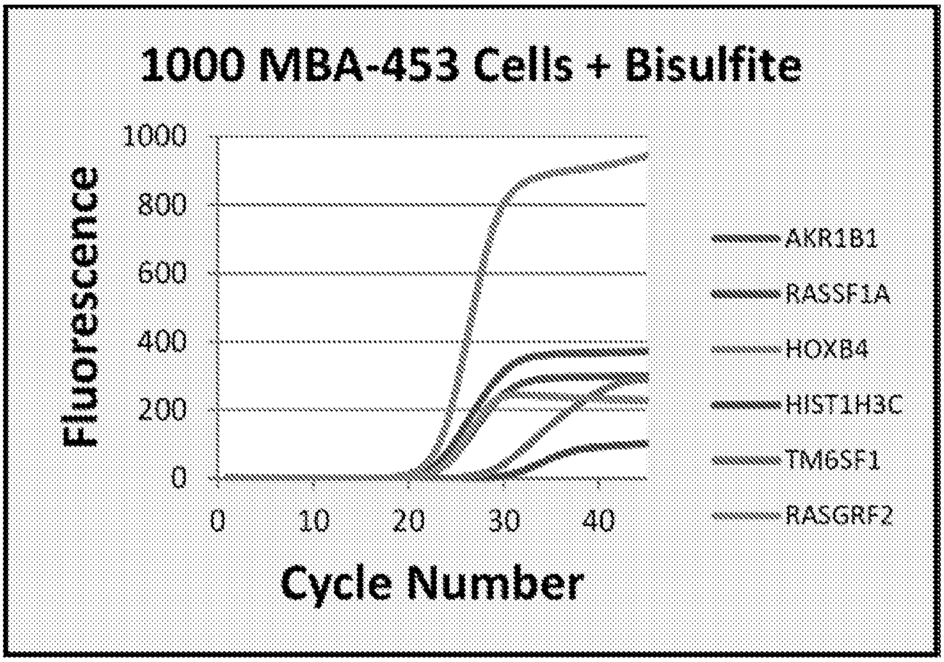


Fig. 15A

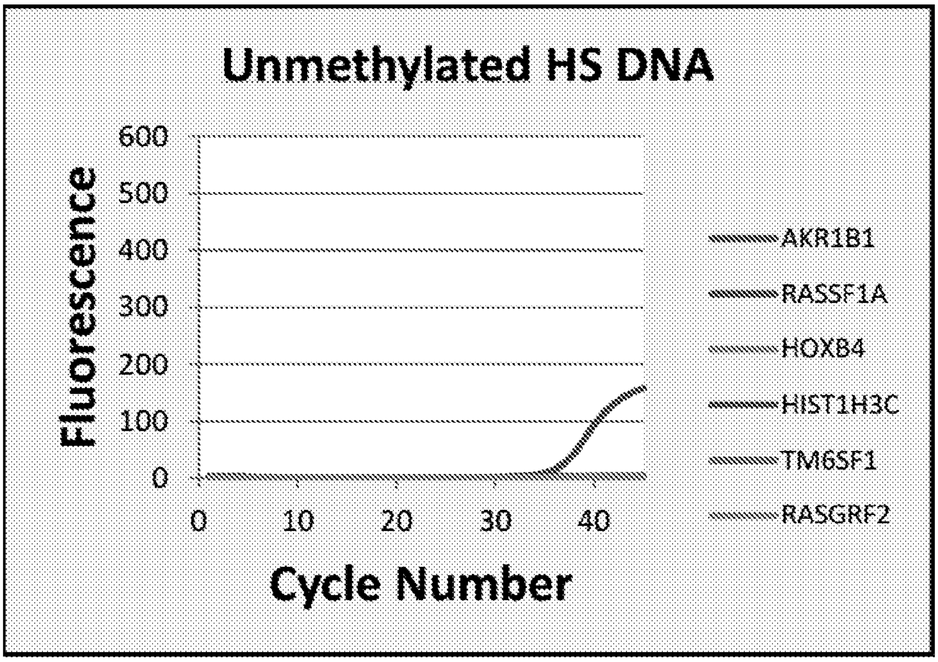


Fig. 15B

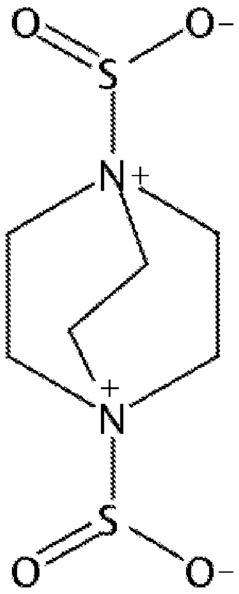

O
S
O−
N+
N+
S
O
O−

Fig. 16

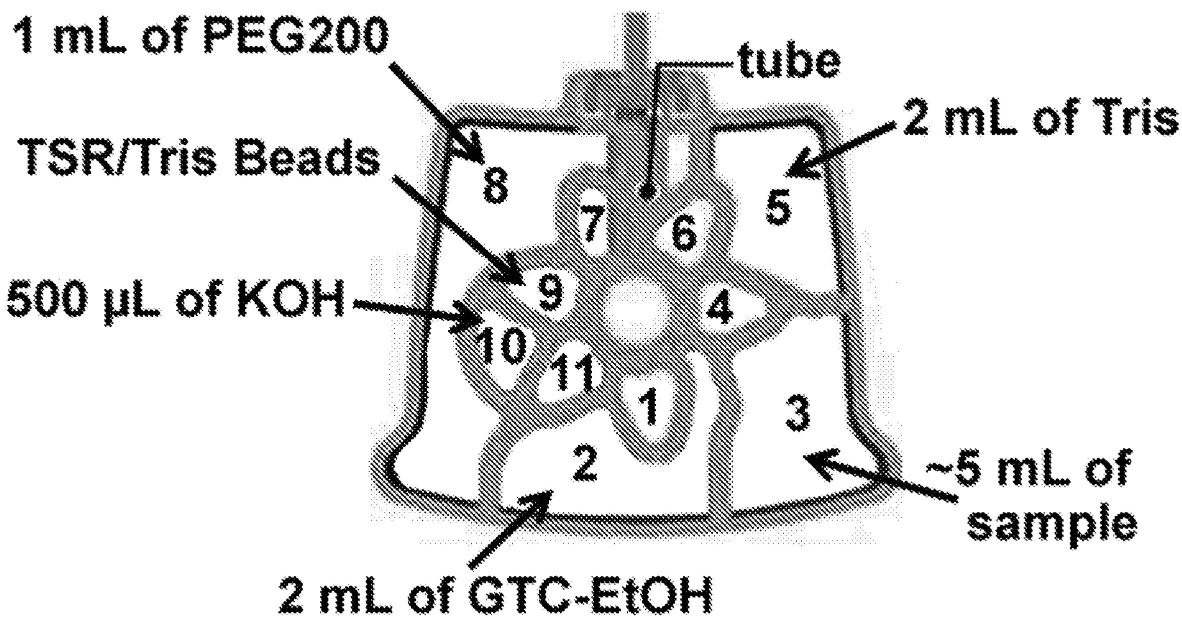
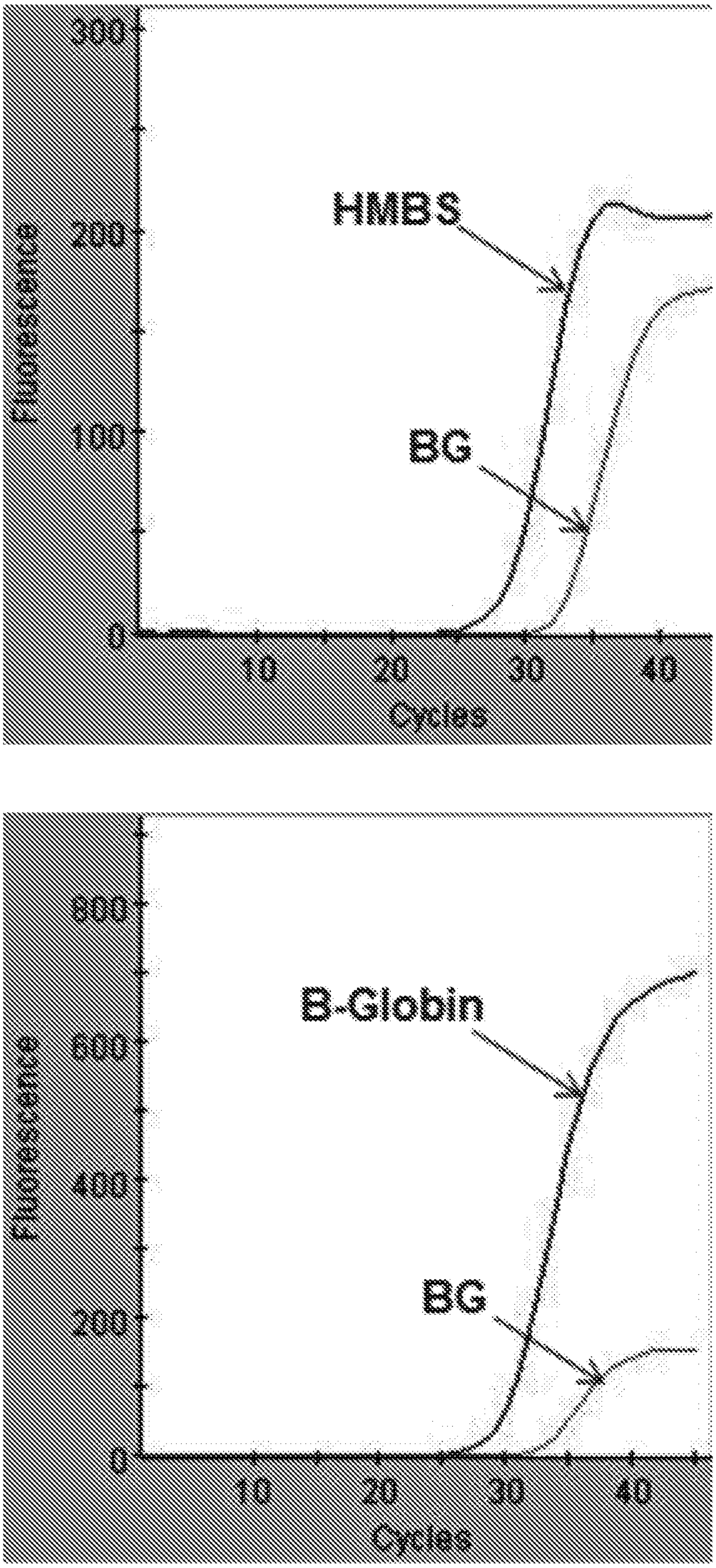

1 mL of PEG200
tube
2 mL of Tris
TSR/Tris Beads
500 µL of KOH
~5 mL of sample
2 mL of GTC-EtOH
1
2
3
4
5
6
7
8
9
10
11

Fig. 17



300
200
100
0
Fluorescence
HMBS
BG
10
20
30
40
Cycles
800
600
400
200
0
Fluorescence
B-Globin
BG
10
20
30
40
Cycles

Fig. 18

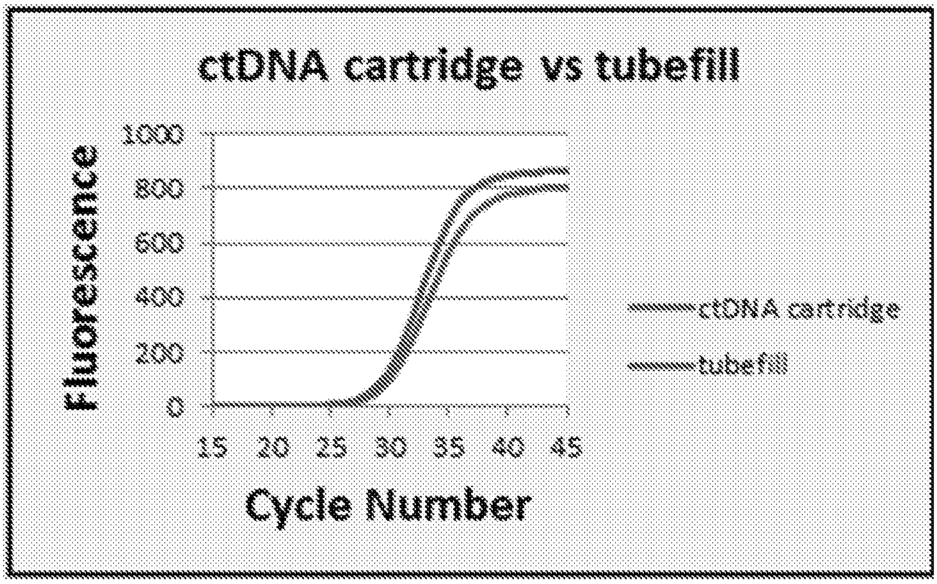


Fig. 19A

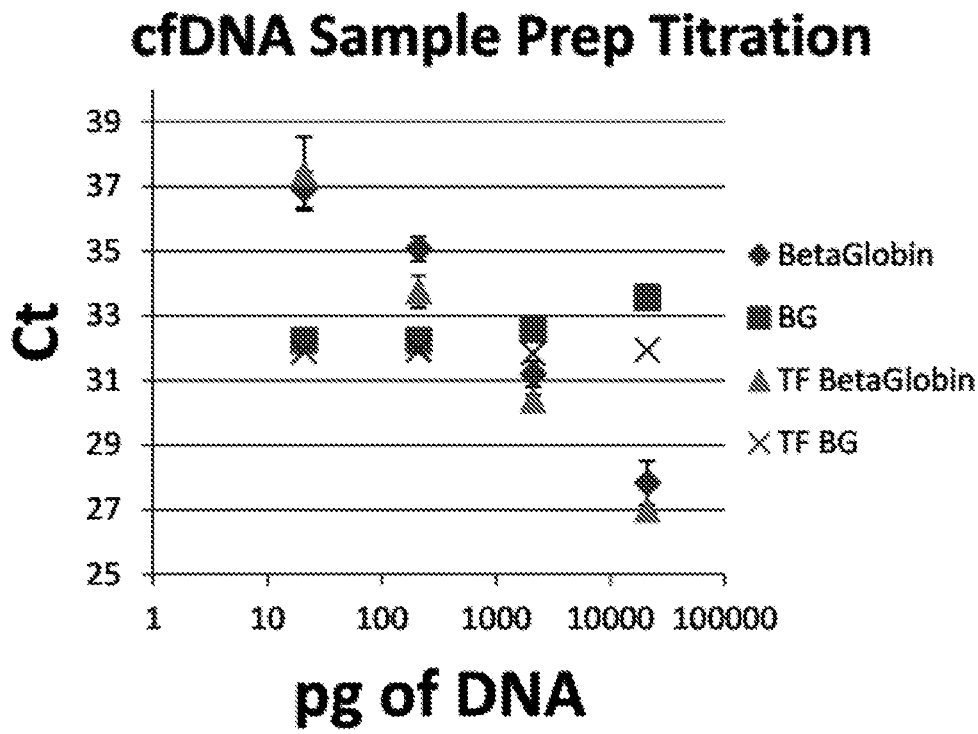


Fig. 19B

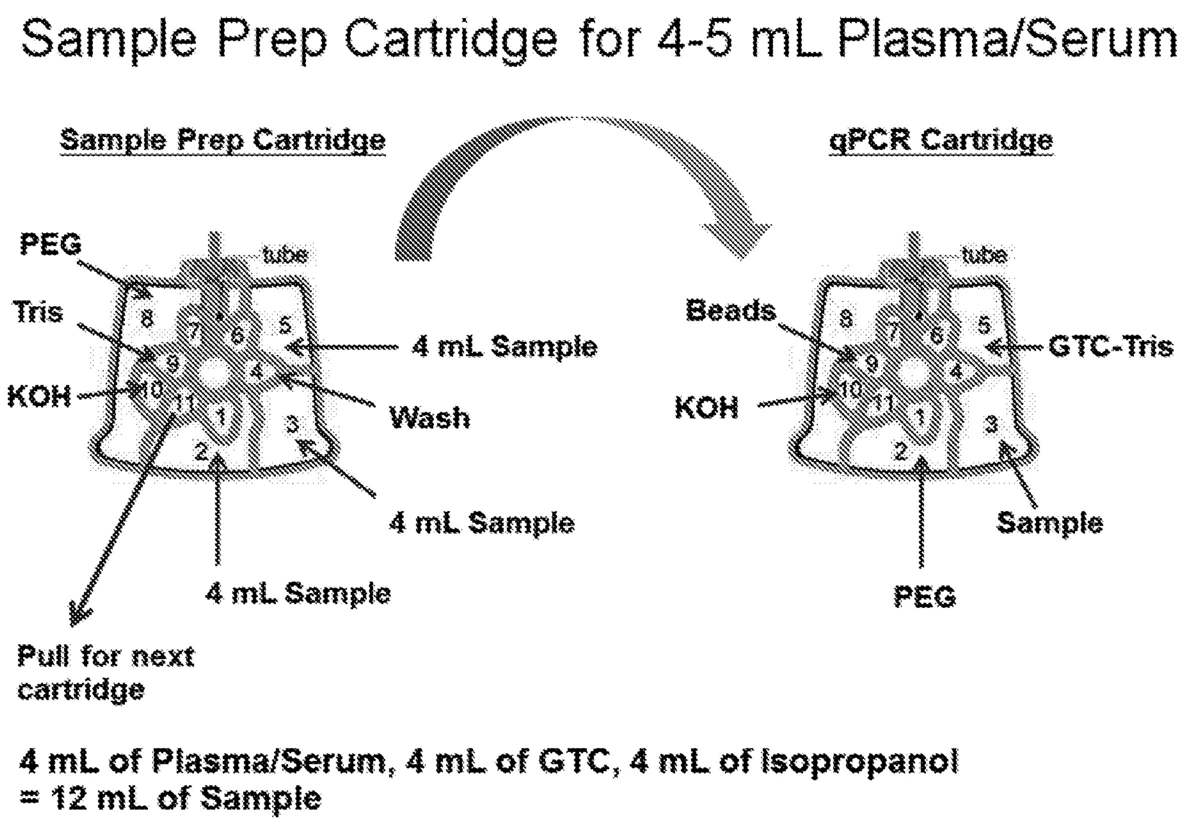


Fig. 20A

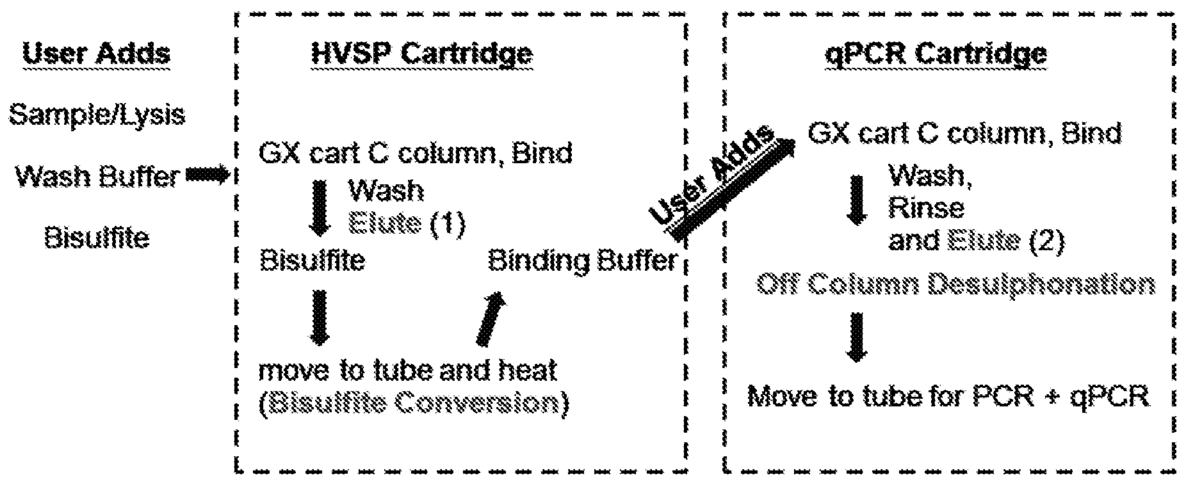


Fig. 20B

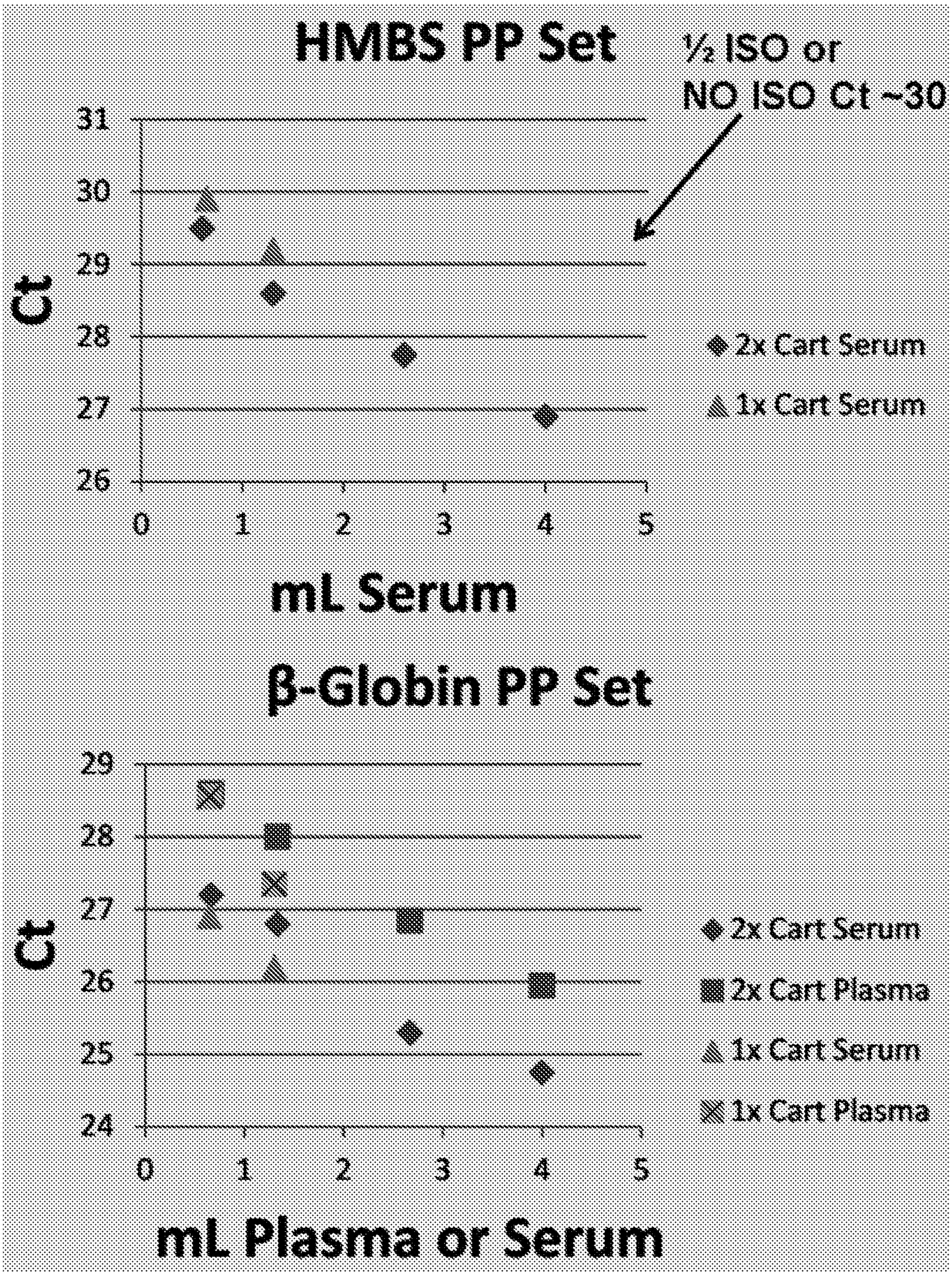

HMBS PP Set
½ ISO or
NO ISO Ct ~30
Ct
31
30
29
28
27
26
0
1
2
3
4
5
mL Serum
2x Cart Serum
1x Cart Serum
β-Globin PP Set
Ct
29
28
27
26
25
24
0
1
2
3
4
5
mL Plasma or Serum
2x Cart Serum
2x Cart Plasma
1x Cart Serum
1x Cart Plasma

Fig. 21

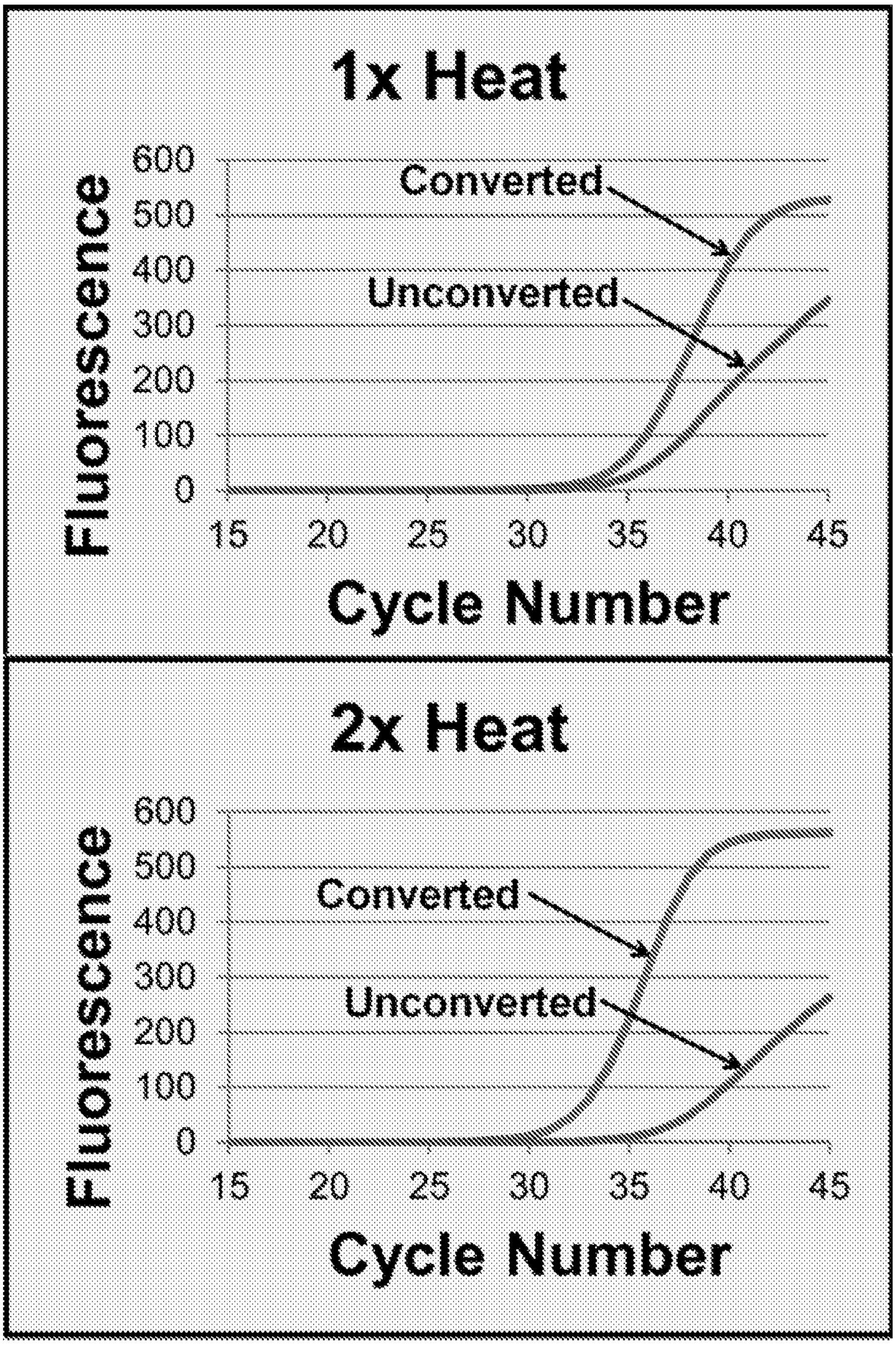

1x Heat
Fluorescence
600
500
400
300
200
100
0
15
20
25
30
35
40
45
Cycle Number
Converted
Unconverted
2x Heat
Fluorescence
600
500
400
300
200
100
0
15
20
25
30
35
40
45
Cycle Number
Converted
Unconverted

Fig. 22

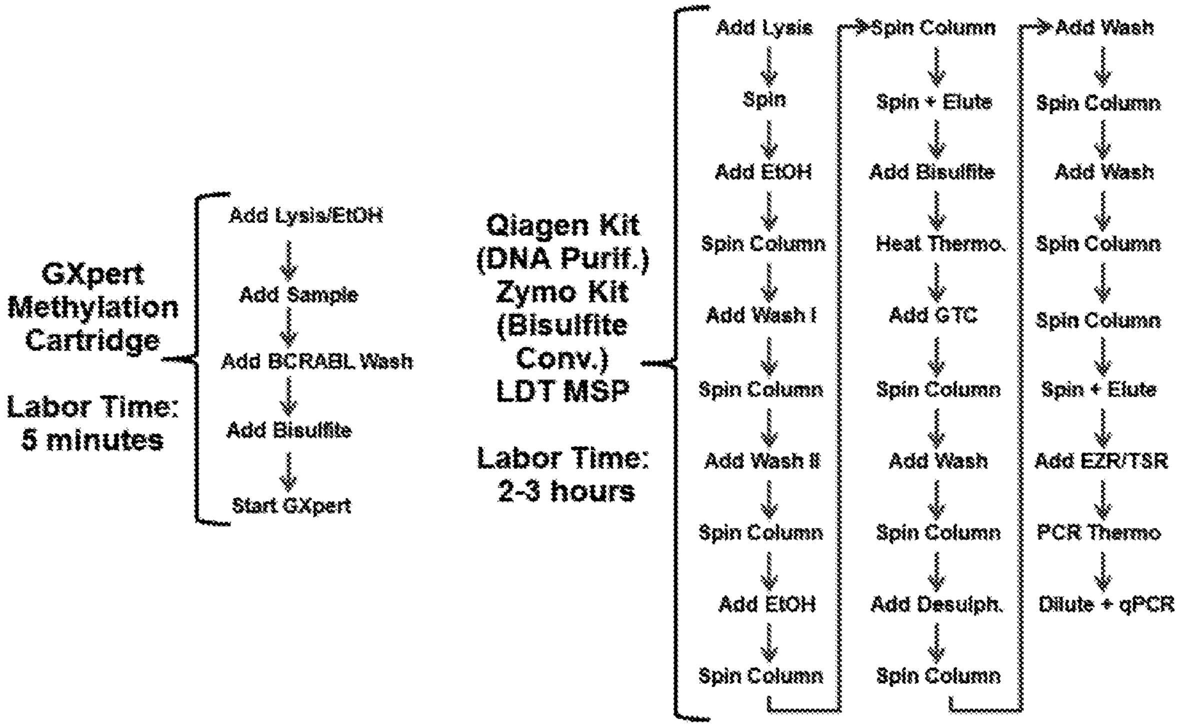

GXpert
Methylation
Cartridge
Labor Time:
5 minutes
Add Lysis/EtOH
Add Sample
Add BCRABL Wash
Add Bisulfite
Start GXpert
Qiagen Kit
(DNA Purif.)
Zymo Kit
(Bisulfite
Conv.)
LDT MSP
Labor Time:
2-3 hours
Add Lysis
Spin
Add EtOH
Spin Column
Add Wash I
Spin Column
Add Wash II
Spin Column
Add EtOH
Spin Column
Spin Column
Spin + Elute
Add Bisulfite
Heat Thermo.
Add GTC
Spin Column
Add Wash
Spin Column
Add Desulph.
Spin Column
Add Wash
Spin Column
Add Wash
Spin Column
Spin Column
Spin + Elute
Add EZR/TSR
PCR Thermo
Dilute + qPCR

Fig. 23A

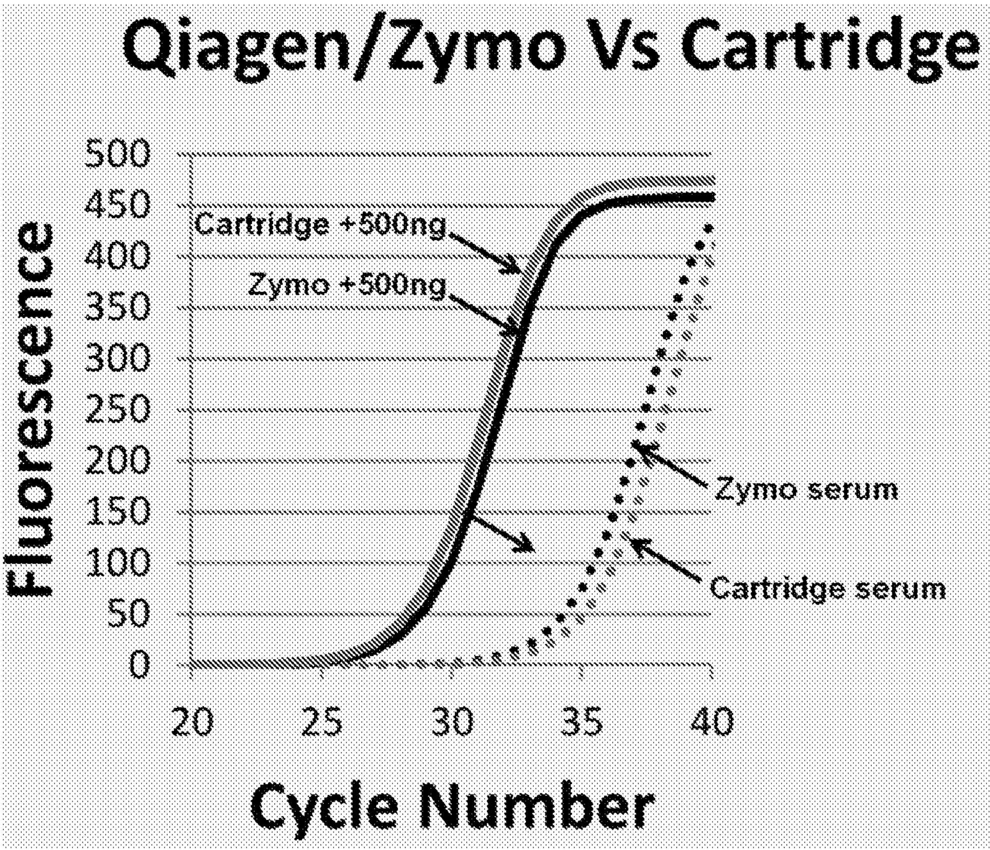

Qiagen/Zymo Vs Cartridge
Fluorescence
500
450
400
350
300
250
200
150
100
50
0
20
25
30
35
40
Cycle Number
Cartridge +500ng
Zymo +500ng
Zymo serum
Cartridge serum

Fig. 23B

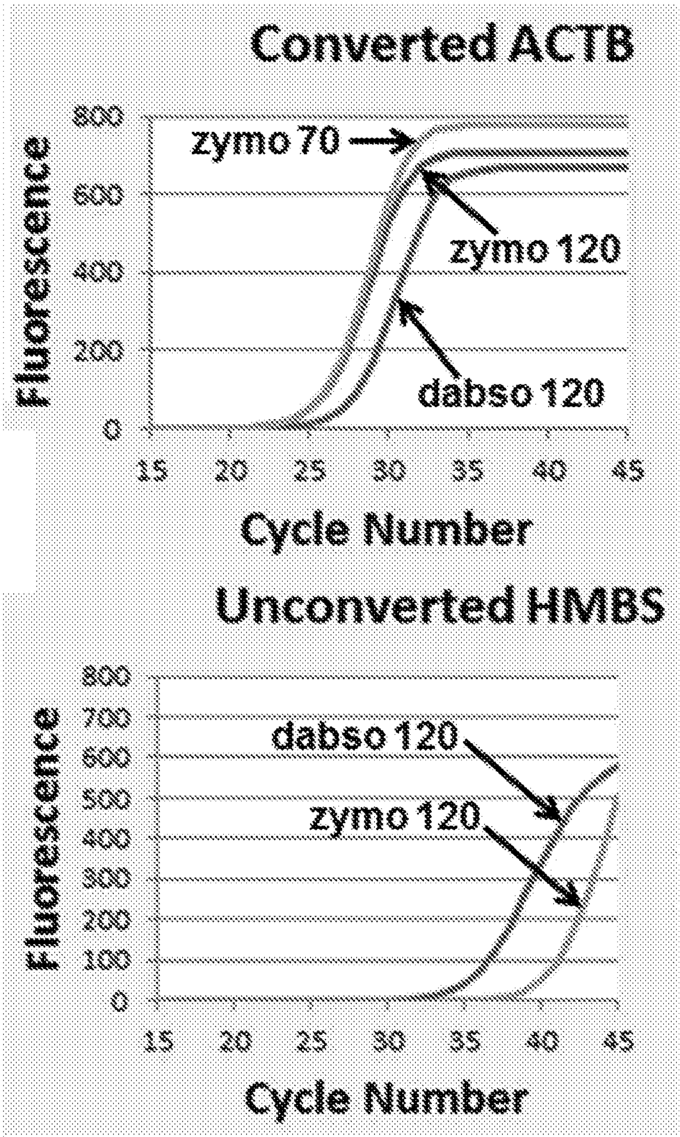

Converted ACTB
Fluorescence
800
600
400
200
0
zymo 70
zymo 120
dabso 120
15
20
25
30
35
40
45
Cycle Number
Unconverted HMBS
Fluorescence
800
700
600
500
400
300
200
100
0
dabso 120
zymo 120
15
20
25
30
35
40
45
Cycle Number

Fig. 24

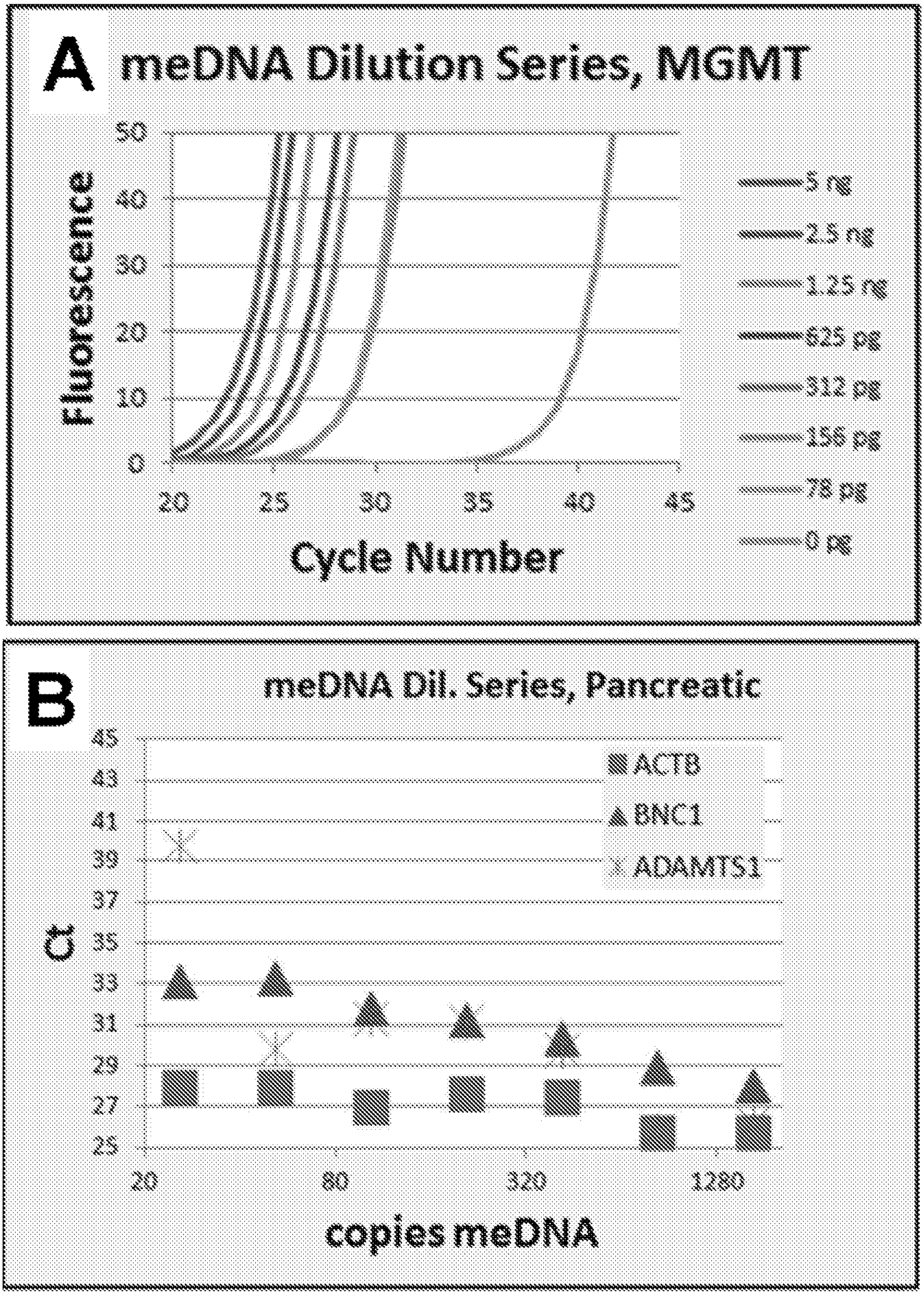

A
meDNA Dilution Series, MGMT
Fluorescence
50
40
30
20
10
0
20
25
30
35
40
45
Cycle Number
5 ng
2.5 ng
1.25 ng
625 pg
312 pg
156 pg
78 pg
0 pg
B
meDNA Dil. Series, Pancreatic
Ct
45
43
41
39
37
35
33
31
29
27
25
20
80
320
1280
copies meDNA
ACTB
BNC1
ADAMTS1

Fig. 25

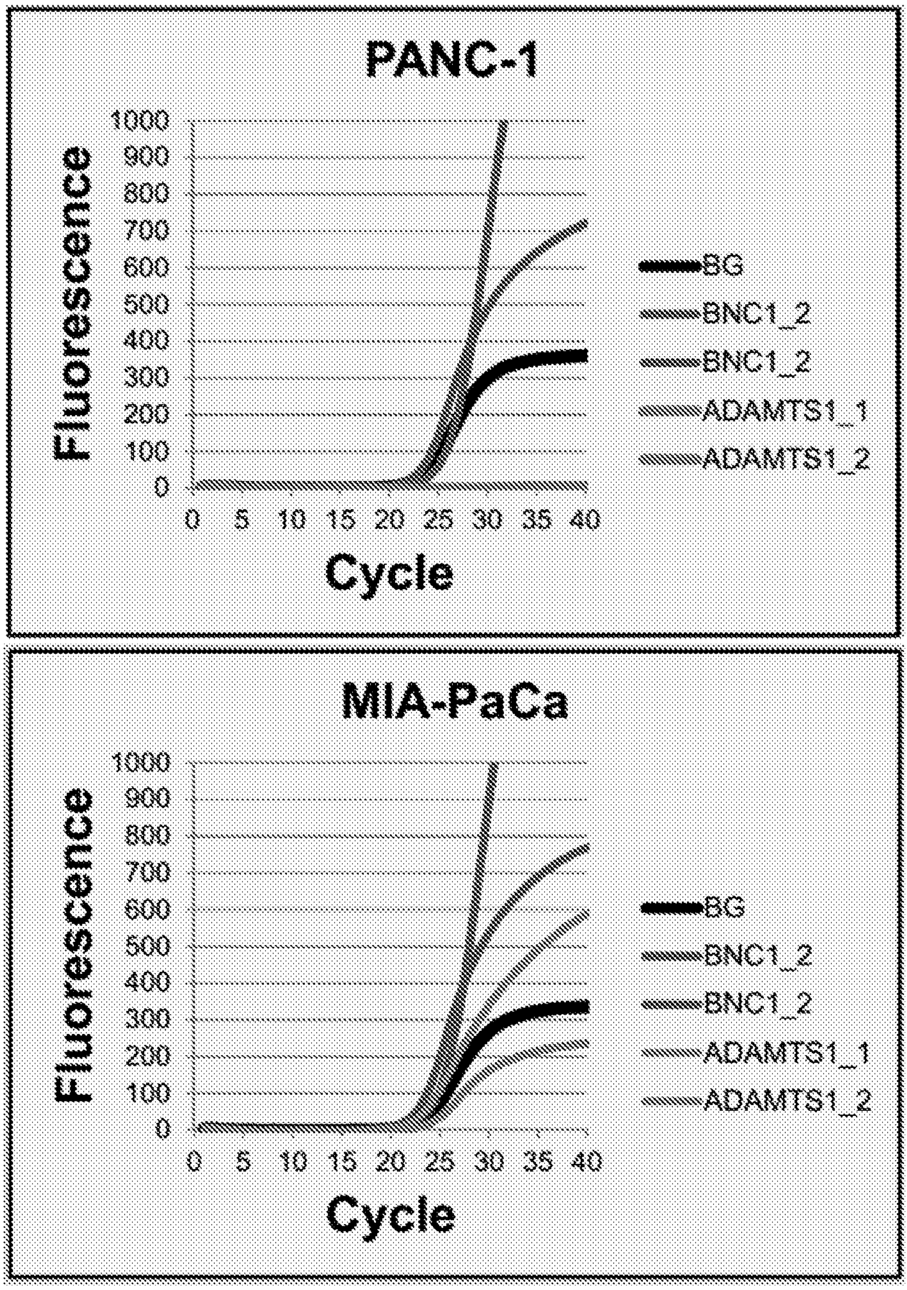

PANC-1
Fluorescence
1000
900
800
700
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40
Cycle
BG
BNC1_2
BNC1_2
ADAMTS1_1
ADAMTS1_2
MIA-PaCa
Fluorescence
1000
900
800
700
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40
Cycle
BG
BNC1_2
BNC1_2
ADAMTS1_1
ADAMTS1_2

Fig. 26

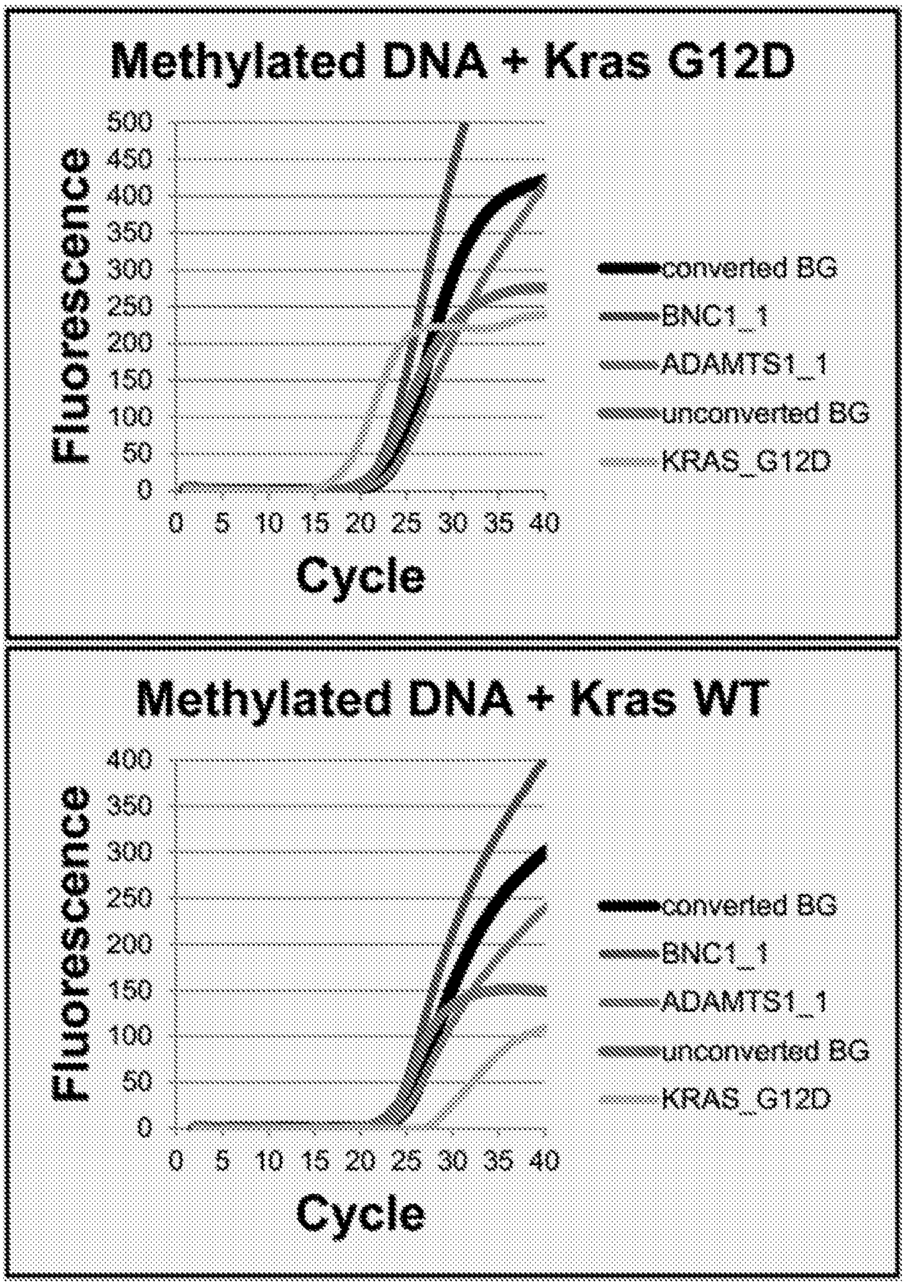

Methylated DNA + Kras G12D
Fluorescence
500
450
400
350
300
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40
Cycle
converted BG
BNC1_1
ADAMTS1_1
unconverted BG
KRAS_G12D
Methylated DNA + Kras WT
Fluorescence
400
350
300
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40
Cycle
converted BG
BNC1_1
ADAMTS1_1
unconverted BG
KRAS_G12D

Fig. 27A

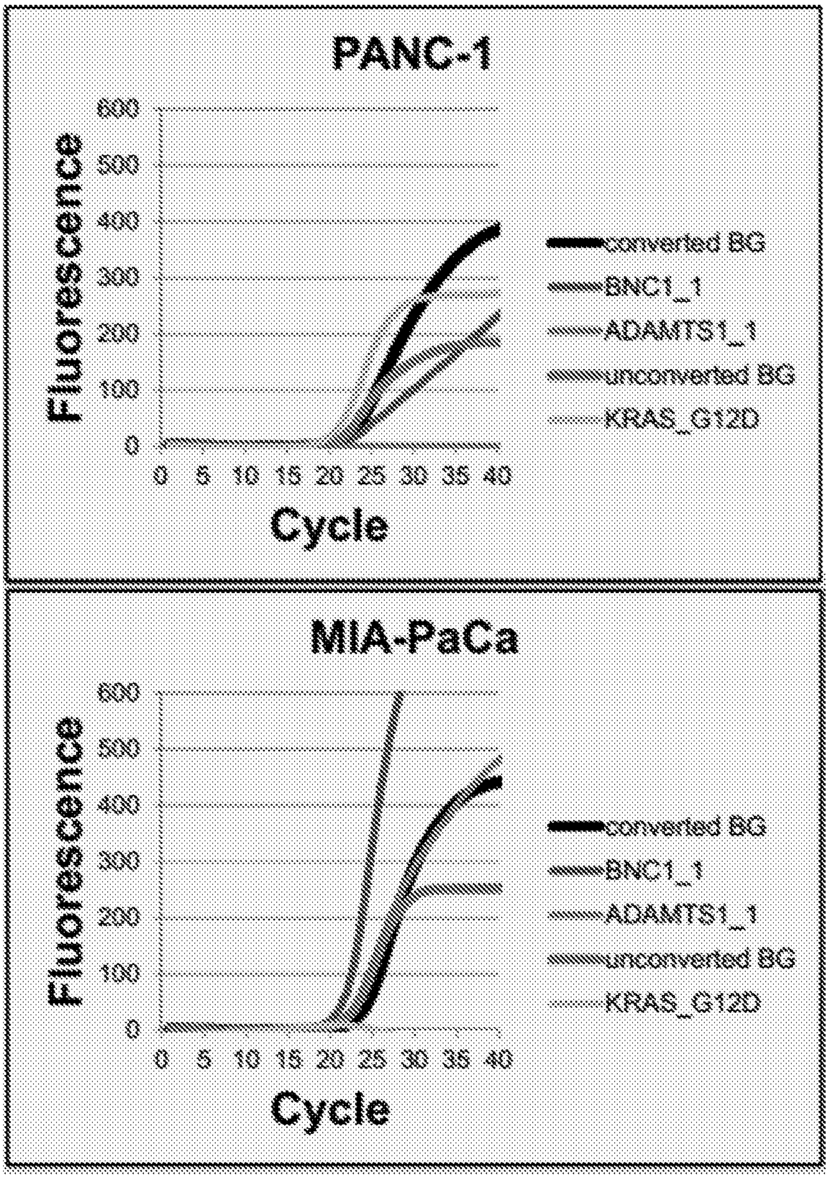
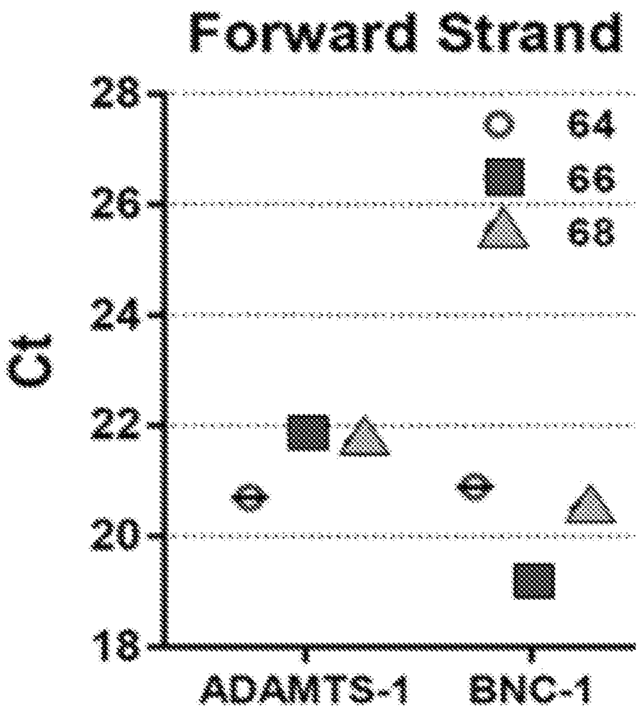

PANC-1
Fluorescence
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40
Cycle
converted BG
BNC1_1
ADAMTS1_1
unconverted BG
KRAS_G12D
MIA-PaCa
Fluorescence
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40
Cycle
converted BG
BNC1_1
ADAMTS1_1
unconverted BG
KRAS_G12D

Fig. 27B


Forward Strand
28
26
24
22
20
18
Ct
64
66
68
ADAMTS-1
BNC-1

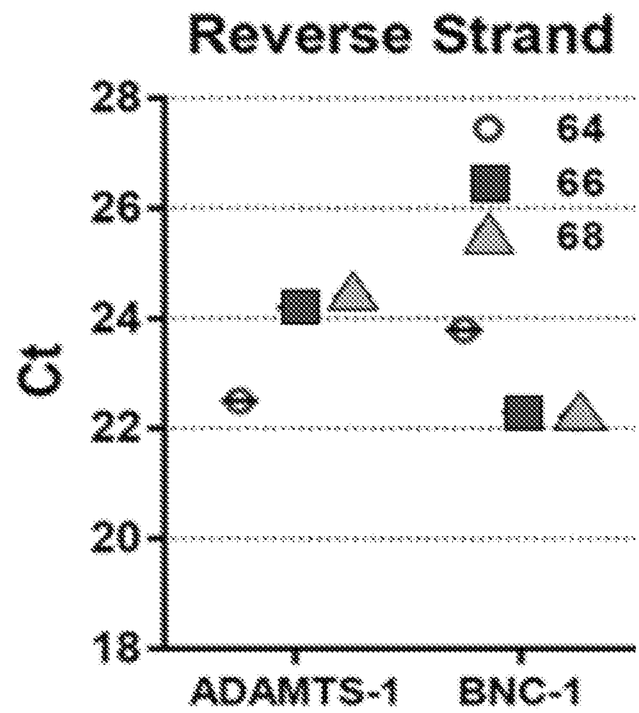
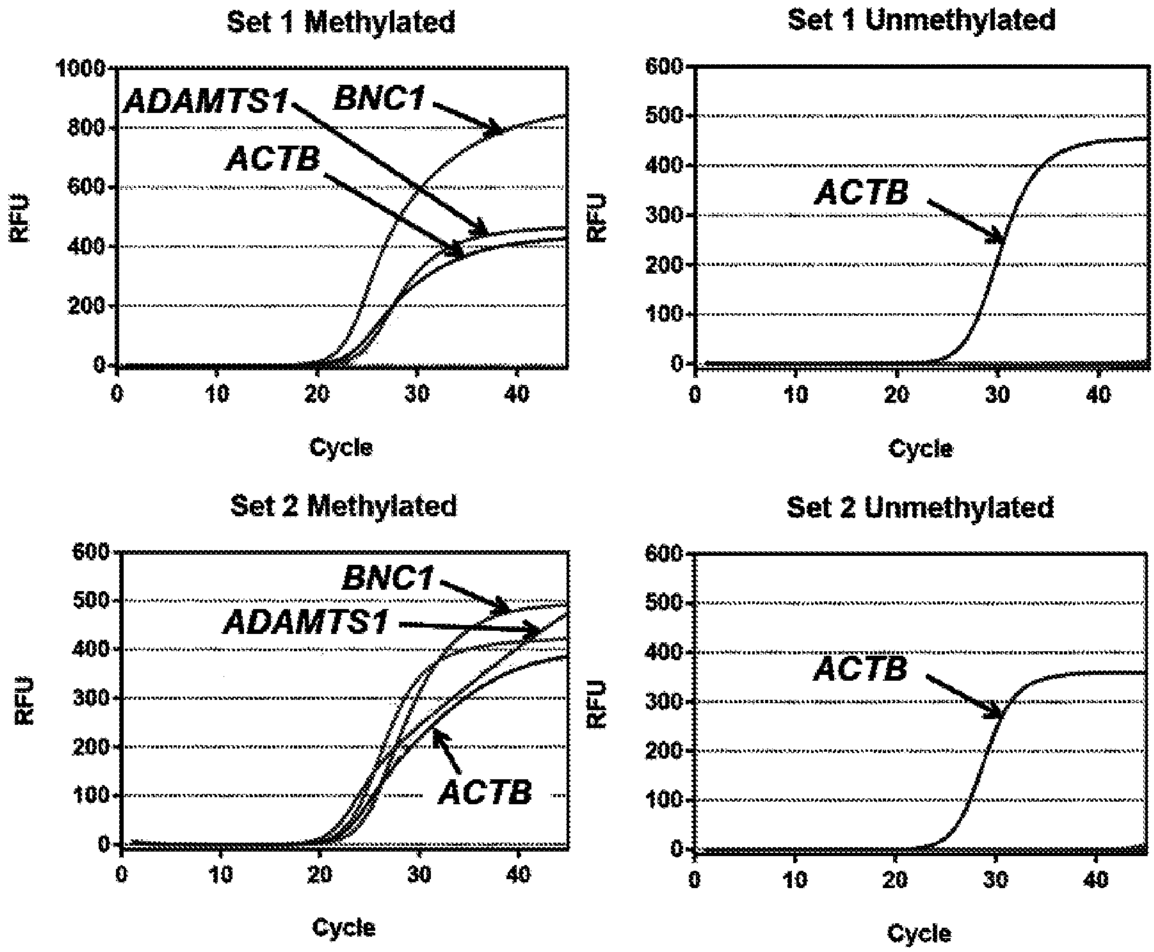

Reverse Strand
28
26
24
22
20
18
Ct
64
66
68
ADAMTS-1
BNC-1

Fig. 28



Set 1 Methylated
RFU
Cycle
ADAMTS1
BNC1
ACTB
Set 1 Unmethylated
ACTB
Set 2 Methylated
BNC1
ADAMTS1
ACTB
Set 2 Unmethylated
ACTB

Fig. 29

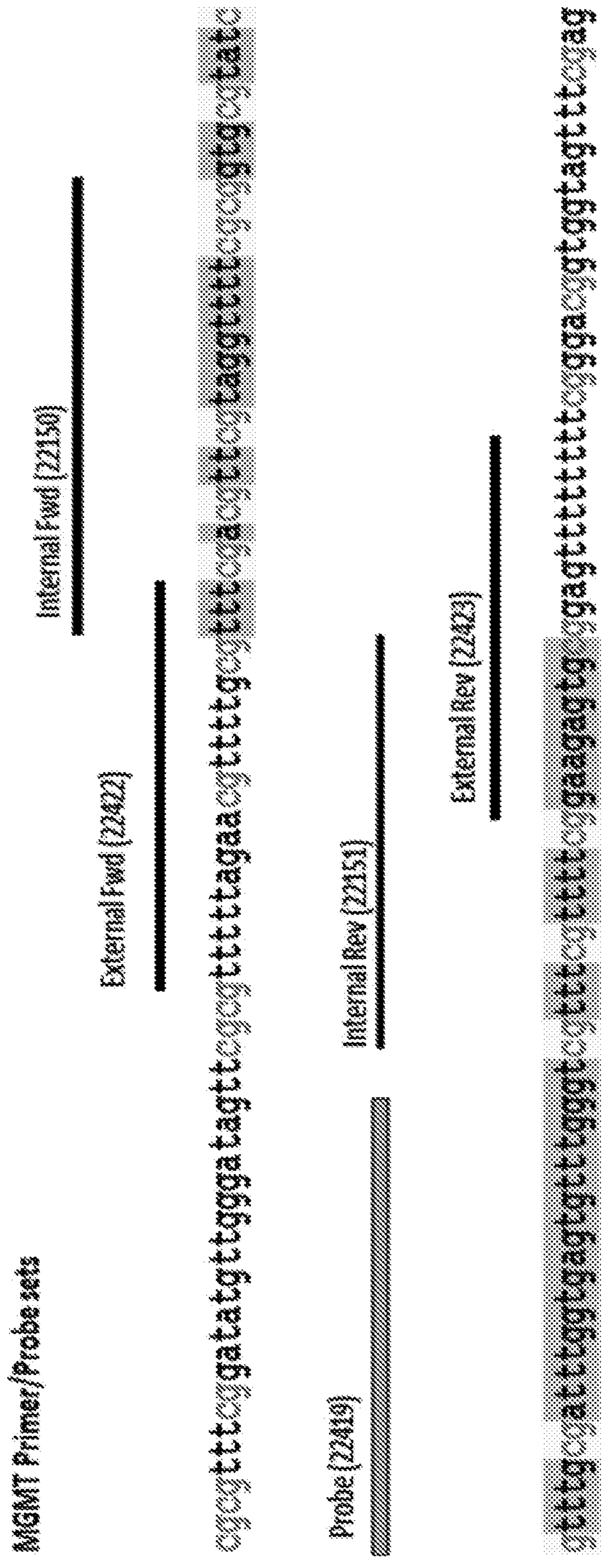
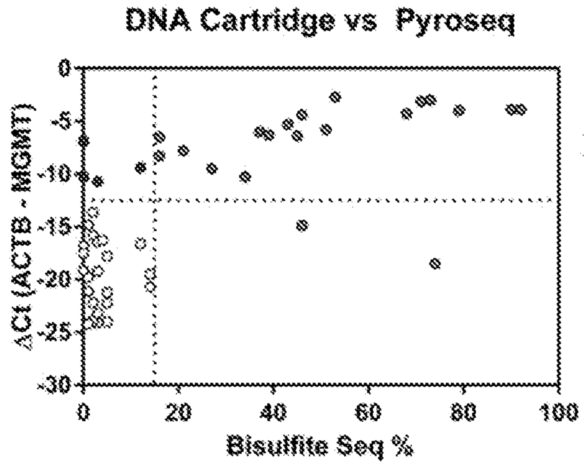
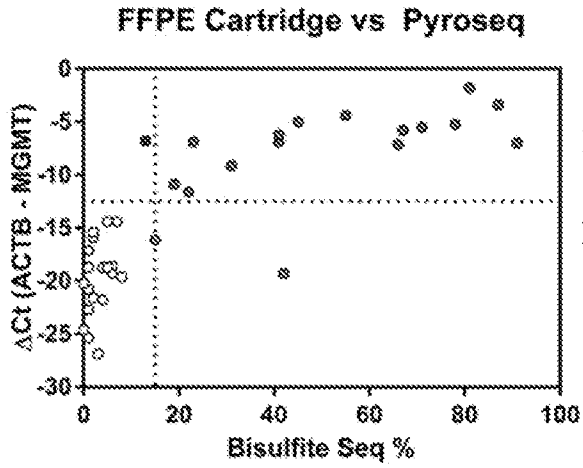

MGMT Primer/Probe sets
Internal Fwd (22150)
External Fwd (22422)
Internal Rev (22151)
External Rev (22423)
Probe (22419)

Fig. 30



	Seq +	Seq -	
Meth +	18	4	22
Meth -	2	24	26
	20	28	48



	Seq +	Seq -	
Meth +	15	1	16
Meth -	2	20	22
	17	21	38

Fig. 31

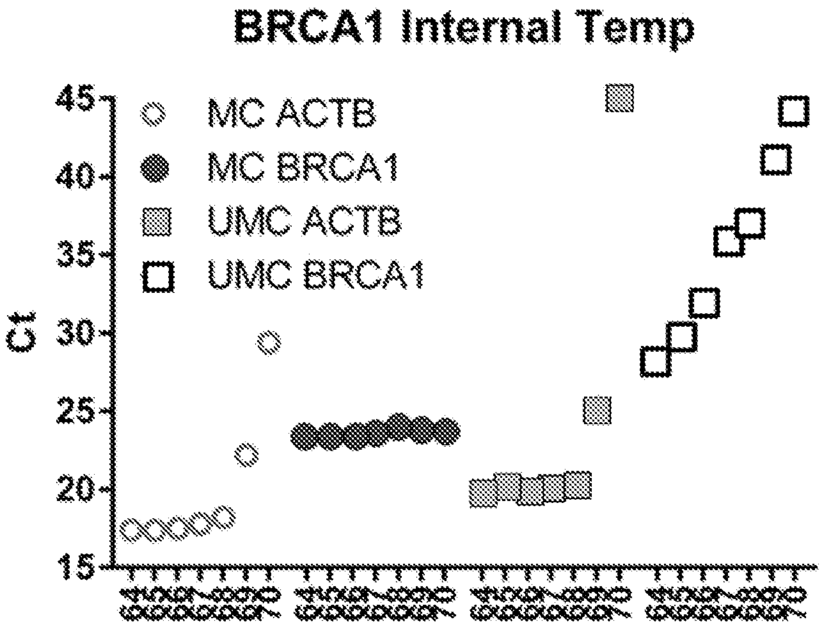

BRCA1 Internal Temp
45
40
35
30
25
20
15
Ct
MC ACTB
MC BRCA1
UMC ACTB
UMC BRCA1

Fig. 32

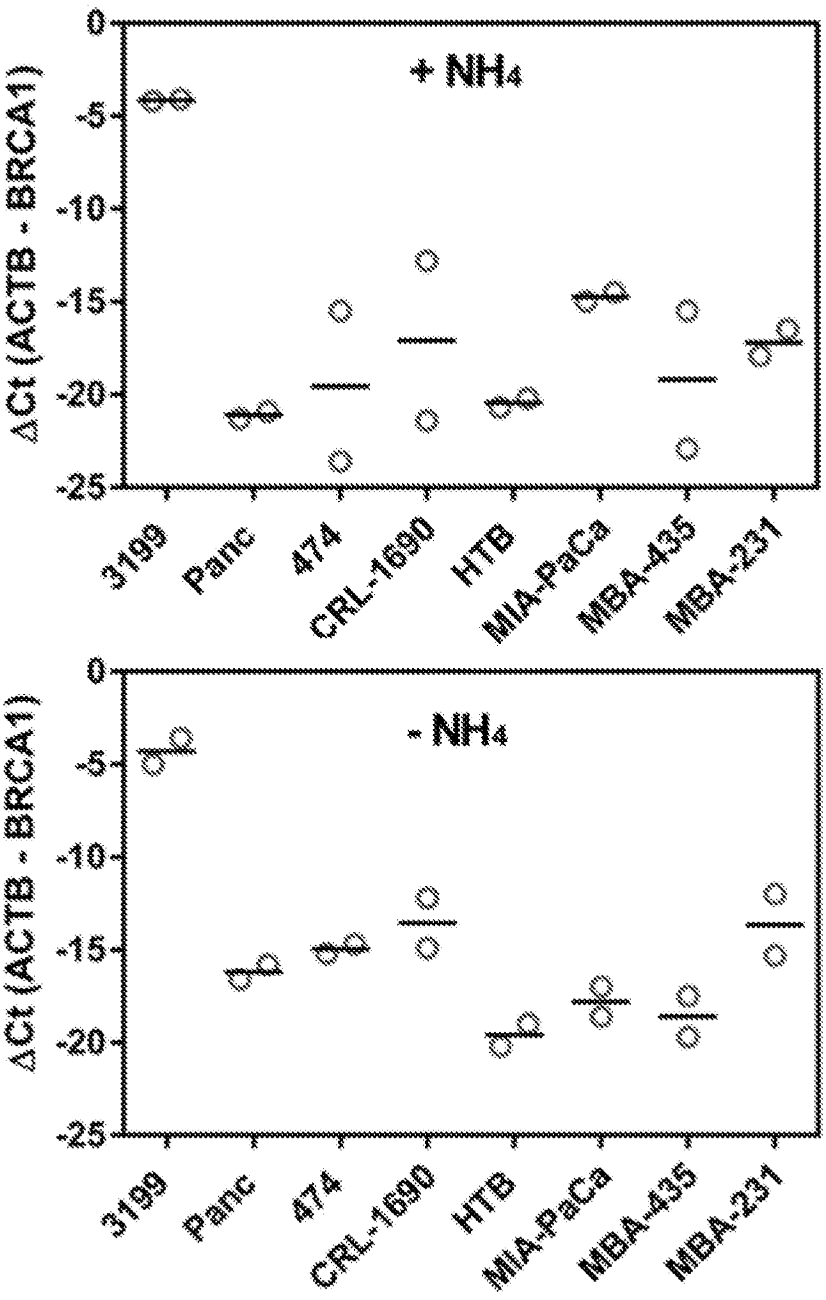

+ NH4
ΔCt (ACTB - BRCA1)
0
-5
-10
-15
-20
-25
3199
Panc
474
CRL-1690
HTB
MIA-PaCa
MBA-435
MBA-231
- NH4
ΔCt (ACTB - BRCA1)
0
-5
-10
-15
-20
-25
3199
Panc
474
CRL-1690
HTB
MIA-PaCa
MBA-435
MBA-231

Fig. 33

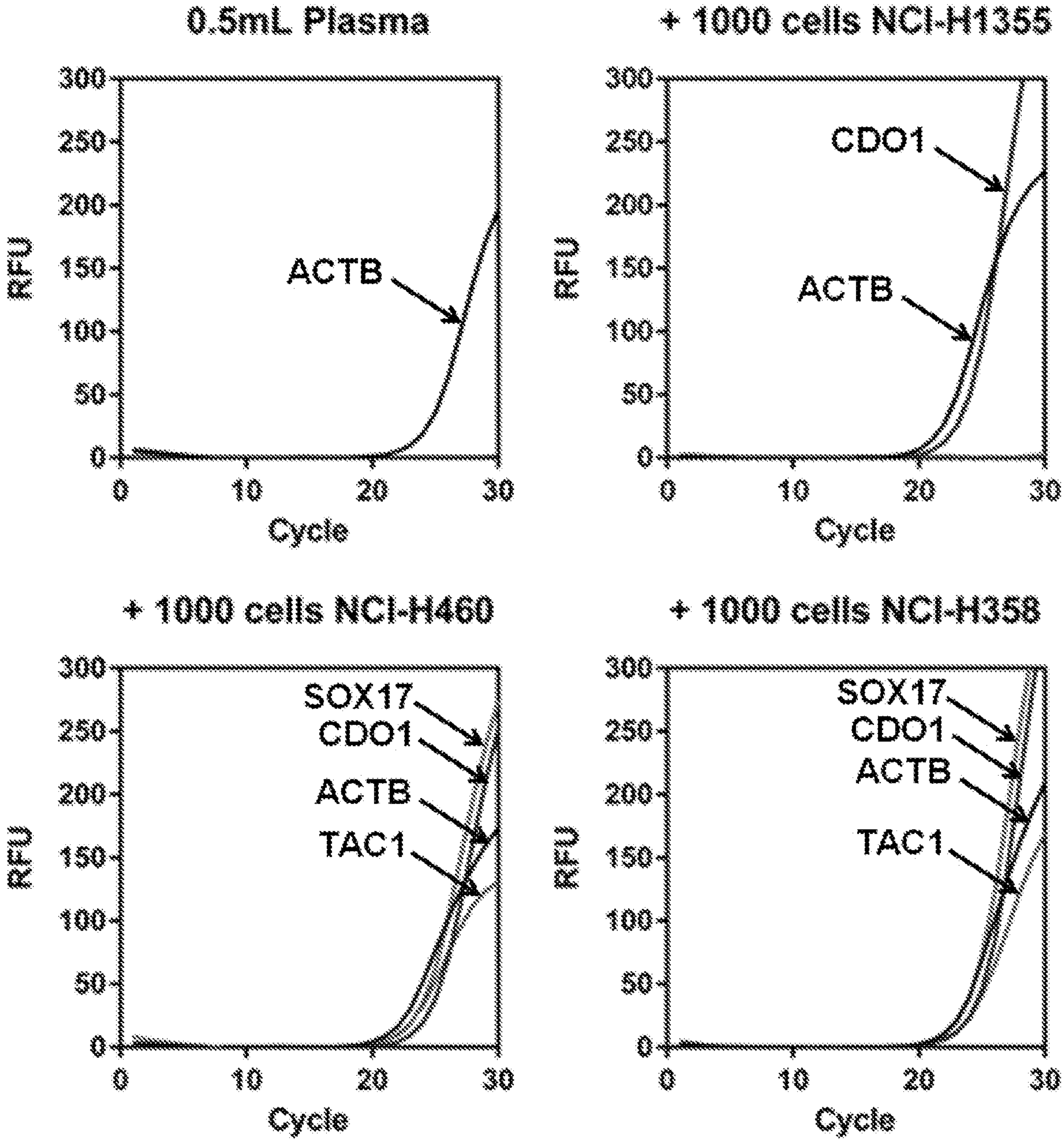

0.5mL Plasma
+ 1000 cells NCI-H1355
+ 1000 cells NCI-H460
+ 1000 cells NCI-H358
RFU
Cycle
0
10
20
30
50
100
150
200
250
300
ACTB
CDO1
SOX17
TAC1

Fig. 34