MethylMeter®: bisulfite-free quantitative and sensitive DNA methylation profiling and mutation detection in FFPE samples

Aim: Development of a sensitive method for DNA methylation profiling and associated mutation detection in clinical samples. **Materials & methods:** Formalin-fixed and paraffin-embedded tumors received by clinical laboratories often contain insufficient DNA for analysis with bisulfite or methylation sensitive restriction enzymes-based methods. To increase sensitivity, methyl-CpG DNA capture and Coupled Absorption PCR Signaling detection were combined in a new assay, MethylMeter®. Gliomas were analyzed for *MGMT* methylation, glioma CpG island methylator phenotype and *IDH1* R132H. **Results:** MethylMeter had 100% assay success rate measuring all five biomarkers in formalin-fixed and paraffin-embedded tissue. *MGMT* methylation results were supported by survival and mRNA expression data. **Conclusion:** MethylMeter is a sensitive and quantitative method for multitarget DNA methylation profiling and associated mutation detection. The MethylMeter-based GliomaSTRAT assay measures methylation of four targets and one mutation to simultaneously grade gliomas and predict their response to temozolomide. This information is clinically valuable in management of gliomas.

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Background

DNA methylation detection methods for genome-level analysis and characterization of specific loci have had extensive use in biomarker discovery and cancer research [1–3]. There has been a slow translation of these methods into clinical tests, however, a major barrier being the need for high sensitivity when processing clinical samples. Most solid tumors are formalin-fixed and paraffin-embedded (FFPE) to allow WHO grading by a pathologist. The fixation process damages the DNA in the sample by causing DNA–DNA and DNA–protein crosslinks. DNA strand lengths are shortened due to the accumulation of strand breaks during fixation and upon long-term storage [4]. Sequence artifacts are also induced that cause conversion of cytosine to thymine [5–7].

The most commonly used DNA methylation detection methods are based on DNA sequence conversion by treatment with sodium bisulfite to convert cytosine, but not 5-methylcytosine, to uracil. Bisulfite-based methods share common drawbacks when applied to clinical testing. DNA conversion induces damage, on top of the damage caused by fixation, causing reduced sensitivity and imposing a requirement for higher sample input. Sequence conversion is difficult to perform completely and reproducibly with FFPE samples [8,9]. Sample loss is possible during sample clean-up to remove residual bisulfite [10]. The reduction in the amplifiable fraction after bisulfite treatment is large enough to require quality control measures for the treated DNA [11].

The bisulfite-dependent methods methylation specific PCR (MS-PCR) and real

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time MS-PCR use primers and probes that overlap with a small number of CpGs. An inference about the methylation level of a relatively large target is based on the success or failure of PCR amplification with each probe set. The accuracy of this approach is adversely affected by heterogeneity in a target where individual molecules have different methylation patterns. Probes that focus on one extreme or the other of the possible methylation patterns are unlikely to generate signals from divergent partially methylated target molecules. Methods that measure bisulfite-induced changes in T_m , like DNA melting curve analysis, can detect methylation heterogeneity because all the CpG sites contribute to the T_m determination; however, methylated DNA quantitation is complicated from melt profiles of heterogeneous samples [12,13].

Bisulfite-free methods avoid the complications associated with DNA sequence conversion. Methylation sensitive restriction endonuclease (MSRE) methods exploit restriction sites containing CpG. MSRE methods rely on enzymes that cleave unmethylated targets but leave fully methylated targets unaffected. The uncleaved methylated DNAs are then amplified. This method is susceptible to incomplete digestion, which is especially acute with FFPE and damaged DNA samples. MSRE methods are also adversely affected by heterogeneity in the methylation pattern [14,15]. For example, the presence of a persistently unmethylated targeted restriction site can cause an otherwise methylated DNA to appear unmethylated.

In spite of these problems, data from FFPE tissues can be obtained in a research setting where there is access to relatively large number of samples, and a significant sample rejection rate due to fixation damage can be tolerated. In the original study utilizing MS-PCR to show that methylation of the *MGMT* gene predicted response to temozolomide, the success rate was only 36%. The study began with 573 blocks of FFPE tumors. Of these, 266 samples were rejected because they contained insufficient DNA for bisulfite treatment. The remaining 307 samples were treated, from which 101 failed to produce results. Ultimately only 206 of 573 samples yielded results for *MGMT* methylation [16]. However, the study produced sufficient data to establish the clinical relationship between *MGMT* promoter methylation levels and drug response.

We have developed a highly sensitive methylation detection method, MethylMeter (Figure 1), which has an extremely low failure rate (<0.1% to date), even with small patient tumors or FFPE tissues that have been stored for over 25 years. MethylMeter first uses a Me-CpG-binding domain (MBD) protein, MethylMagnet [17], to separate methylated DNA from unmethylated DNA, similar to the methylated-CpG

island recovery assay (MIRA) [18]. Detection sensitivity is achieved with a novel abortive transcription (Abscription®)-based signal generation process to measure methylation levels of specific targets.

MethylMagnet is a recombinant MBD2 Glutathione-S-transferase (GST)-fusion protein which exists as a dimeric MBD due to the dimerization of the GST domain [19,20]. It is unaffected by the sequence context of a methylated CpG site [21–23]. Dimerization is expected to increase affinity to methylated DNA based on results with concatemeric forms [24,25]. MethylMagnet takes advantage of the high specificity of the MBD2 methyl-CpG binding domain and its bias for clustered CpGs, which favors the analysis of CpG islands [21,26]. Because all methylated CpGs in a fragment can contribute to binding, the assay is less affected by heterogeneity of the methylation pattern. After separation, methylated and unmethylated targets are amplified and tagged for Abscription by PCR of each fraction. The PCR step plays no role in the discrimination between methylated and unmethylated targets, so there are fewer constraints on primer development than there are for bisulfite-based methods.

The high sensitivity of MethylMeter is achieved by using Abscription for detection in a process called CAPS™ (Coupled Abscription PCR Signaling), which we have shown previously to be two to three orders of magnitude more sensitive than Taqman quantitative realtime-PCR (qPCR)-based detection [17]. Abscription involves the reiterative synthesis of short ‘abortive’ RNA transcripts by an RNA polymerase that remains bound at the promoter. Although this process occurs naturally at low turnover with many RNA polymerases, by manipulating the DNA promoter sequences, we have developed several abortive promoter cassettes (APCs), each designed to generate multiple copies of a single, specific, short abortive RNA transcript (abscrip) per minute. The APCs are attached to the amplicons generated from the DNA targets in the methylated and unmethylated fractions, and the amount of each abscrip produced is a measure of the amount of the original DNA target in each fraction. The signal amplification provided by Abscription allows detection of as little as 20 picograms of DNA in 36 cycles, reducing the non-specific amplification associated with preamplification and higher cycle numbers.

A MethylMeter-based test, GliomaSTRAT, was developed to simultaneously grade gliomas by determining CpG island methylator phenotype (CIMP) using the methylation level of three genes and; determine the tumor’s response to first-line chemotherapy with Temozolomide by measuring *MGMT* promoter

Figure 1. MethylMeter overview. (A) Workflow and timeline for MethylMeter. Genomic DNA is cut with a restriction enzyme that is not affected by CpG methylation to separate CpG islands from one another. The DNA is then separated into methylated DNA (Me, beads) and unmethylated DNA (U, supernatant fraction) with glutathione magnetic beads containing a GST – methyl CpG-binding domain fusion protein, MethylMagnet® (step 1). Using CAPS, individual gene CpG islands are amplified from each fraction during which an APC, the binding site for an RNA polymerase, Abscriptase®, is added (Part A, step 2a). Methylated DNA can be amplified directly on the beads. A ribonucleotide initiates Abscription® (Part A, step 2b). The dinucleotide is elongated by the addition of one or more ribonucleoside triphosphates (rNTPs) encoded by the APC to generate multiple (n) Abortive RNA Transcripts (3- or 11-nt long), or Abscripts, per target amplicon. Abscripts are quantified in both the methylated and unmethylated fractions using either mass spectrometry or abscript-dependent molecular beacon fluorescence. The amount of Abscript is a measure of the amount of DNA target present in the starting sample, so can be used to determine the percentage methylation for that target. (B) CAPS for mass spectrometry versus fluorescent beacon-based detection. The amount of a specific targeted CpG island on the beads (Me) and in the supernatant fraction (U) is measured by CAPS. Targets are amplified by PCR to attach an APC to target amplicons (Part B, step 1). Conversion of the APC from a single-stranded form to a duplex form activates it for Abscription. Each APC encodes a different Abscript. Abscription is initiated by addition of Abscriptase® (RNAP) and ribonucleotides that are specific for the attached APC (Part B, step 2). There are two methods to measure the Abscription reaction, depending on the Abscript size. In example Part B, step 2a, the attached APC encodes the trinucleotide UpUpG, so addition of the dinucleotide initiator UpU and GTP produces the trinucleotide UpUpG. In example Part B, 2b, the APC encodes an 11-mer, and Abscription requires pApU, GTP, UTP and CTP to produce the Abscript pApUpCpGpUpGpCpUpUpU. Trinucleotide abscripts are detected directly by LC–MS (Part B, 3a). The 11-nt abscripts are detected indirectly by Abscript-mediated molecular beacon opening (Part B, 3b & Figure 2). Because Abscription is reiterative, multiple Abscripts (n) are produced per minute per APC. Abscription®: Abortive transcription; APC: Abortive promoter cassette; CAPS: Coupled Abscription PCR Signaling; GST: Glutathione-S-transferase; RNAP: RNA polymerase; rNTP: ribonucleoside-5'-triphosphate.

methylation. We internally validated this assay by measuring the DNA methylation of two regions of the *MGMT* promoter CpG island and three CpG islands that determine the glioma CIMP (G-CIMP) [27]. The initial G-CIMP assay utilized an eight-gene DNA

methylation profile to determine whether the tumor is a low-grade glioma or high-grade glioma, independent of WHO grade. We have reduced the original eight-gene G-CIMP panel to three genes with 100% agreement with the eight-gene profile and validated

Figure 2. Abscript detection by molecular beacon fluorescence. (A) The 11-mer abscrip (abortive transcripts) can be quantified based on their ability to participate in molecular beacon activation. The molecular beacon has a fluorescein attached to the 5' end of the terminal duplex inverted repeat. The quencher BHQ1 is located at the 3' end. In the closed configuration, any emission of the fluorescein is absorbed by the adjacent quencher and there is no measurable fluorescence. The 11-nt abscrip (dashed line) generated from an amplicon – associated APC, anneals to a single-stranded complementary region in the loop of the molecular beacon. Primer extension of the abscrip by the Coupled Abscription PCR Signaling DNA polymerase generates a more rigid double-stranded product and opens the beacon so that the fluorescein and quencher are separated, resulting in measurable fluorescence emission. (B) Shows plots of FAM in relative fluorescence units as a function of Absorption time for a set of calibrator DNAs. (C) Is a plot of calibrator concentration as a function of the calibrator rates of fluorescence increase. The resulting calibration formula is used to quantify sample DNA concentrations from fluorescence increase rates.
APC: Abortive promoter cassette; FAM: Fluorescein fluorescence increase.

this panel on the original FFPE samples (data not shown).

We corroborated the G-CIMP methylation results with a CAPS assay for the *IDH1* R132H mutation shown to cause CIMP [28]. The assay simultaneously measures the normal wild-type (WT) allele and a ratio of mutation to WT is generated. It has become common practice to determine the *IDH1* and *IDH2* mutational status to infer CIMP, presumably because existing methods for DNA methylation detection lack the sensitivity to reliably measure the methylation of a multigene panel in FFPE samples. Results for *MGMT* methylation on FFPE samples are compared with those generated using bisulfite-free methylation sensitive restriction enzymes (MSRE) or bisulfite-based, mass spectrometry (MS)-based EpiTyper on RCL2-fixed samples from the same tumor. The distinction between the fixation methods is significant because RCL2 fixed samples suffer less damage than the FFPE fixed counterparts.

We describe here two versions of the MethylMe assay and its application to the analysis of clinical samples. In Format I, the abscipt produced is a trinucleotide that is detected by MS. This method has high multiplexing potential, but requires access to a mass spectrometer not available in many laboratories. In Format II, the abscipt produced is an 11-mer RNA that hybridizes to and primes the opening of a fluorescent molecular beacon and can be measured on a qPCR or other fluorescent instrument [29].

Materials & methods

Tissue & DNA samples

FFPE and RCL2-fixed tissue sections [30] from newly diagnosed gliomas were provided by the Austrian Institute of Technology, Institute of Neuropathology, Medical University of Vienna and NuvOx Pharma (AZ, USA) and included tumors that had been assigned grade 2, 3 or 4 via histopathology. Tumor DNAs from frozen recurrent glioblastoma multiforme (GBM) were provided by Tocagen, Inc. (CA, USA).

DNA purification from FFPE tumor sections

FFPE tissue sections were deparaffinized in xylene in two 5 min incubations at 50°C. Solvent changes were made by centrifugation at 14,000 × *g* for 5 min. Residual xylene was removed with a 100% ethanol wash. The tissue pellets were air-dried and suspended in 50 mM Tris-HCl (pH 8.5 at 10 mM and 25°C), 100 mM NaCl, 1 mM EDTA, 0.5% v/v Tween-20, 0.5% w/v NP40, 20 mM DTT and 500 µg/ml Proteinase K. The samples were incubated overnight at 55°C followed by a 20 min incubation at 80°C to inactivate the Proteinase K and to reverse crosslinks.

Nucleic acids were precipitated with half volume of 7.5 M ammonium acetate and three volumes of 100% ethanol. The pellets were dissolved in 50 µl of TE buffer.

DNA fragmentation

DNA was digested with ten units of AluI in 20 mM Tris-HCl pH 8, 50 mM KCl, 2.8 mM MgCl₂ for 1 h to overnight. The extent of cleavage was determined by CAPS as described below. Each cleaved DNA sample and an uncut portion of each reaction was amplified with a primer pair that is sensitive to AluI cleavage due to an AluI site located between the priming sites in the *HFE* CpG island (Figure 3).

Copy number & amplifiable DNA determination

The number of genomic copies/µl was determined for the cut and uncut samples based on linear fluorescence increases during Abscription from a titration of uncut HeLa DNA that was amplified in parallel with the samples. The fraction of cleaved AluI test sites was expressed as 1-(DNA digest signal/uncut control signal). The uncut DNA concentration was taken as the amplifiable DNA content of each sample. The A₂₆₀ was used to calculate the total nucleic acid concentration of each sample. AluI digests were frozen at -20°C without purification.

Methylated DNA fractionation

DNA samples were fractionated using the MethylMagnet® kit (RiboMed CA, USA, cat# MM101K) following the standard protocol except that a 10× binding buffer was used to minimize the dilution of the samples. AluI-cut DNA was mixed with 2 µl of 10× binding buffer (100 mM Tris-HCl pH 7.6 [at 10 mM and 25°C], 1.6 M NaCl, 1 mM EDTA, 0.8% v/v TritonX-100 and 40% v/v glycerol) and a variable amount of DNA dilution buffer (20 mM Tris-HCl pH 8, 50 mM KCl and 2.8 mM MgCl₂) to give a 20 µl binding reaction. The MgCl₂ component (final concentration 2.5 mM) was provided by the DNA sample and the dilution buffer. Amplifiable FFPE DNA inputs ranged from 3 to 20 ng.

Magnetic beads bearing the MethylMagnet GST-MBD2 fusion protein were distributed to 1.7 ml microcentrifuge tubes and were washed with 100 µl of Wash Buffer 1 without prior removal of the bead storage buffer. Upon removal of the wash buffer, the beads were suspended in 20 µl DNA samples in 1× binding buffer and were incubated for 1 h at room temperature (22°C) at 1000 rpm in a thermomixer (Eppendorf, NY, USA). At the end of the binding reaction, the beads were pelleted with a magnet and

Figure 3. Primer sequences. The relationships among the four Coupled Abscription PCR Signaling primers used to make the 11-mer generating APC are shown in the diagram. Forward and reverse target-specific primers are linked at their 5' ends to an APC-DN (downstream) segment (forward primer, A & B) and a UR segment F to the reverse primer C & D. The APC-DN segment encodes the downstream segment of a truncated inactive APC. When the target primers and appended segments are copied in Phase 1 of the PCR program, complements of the APC completion and the universal reverse primers are created. In Phase 2 of the PCR program priming a B–D tagged amplicon with the APC completion primer (E) creates a full-length functional APC. The universal reverse primer is designed to use the same PCR conditions as the APC completion primer. Use of the universal reverse segment simplifies primer design. The table shows sequences of the target primers and their linkages to the APC-DN segment and the universal reverse segment. APC: Abortive promoter cassette; DN: Downstream.

the supernatant fraction containing the unmethylated DNA was recovered. The bead pellets were washed twice with 400 µl of Wash Buffer 2 for 5 min at room temperature (22°C) and at 1000 rpm in a thermomixer. The stringency of Wash Buffer 2 was 300 mM NaCl. A third 400 µl wash with TE buffer (10 mM Tris-HCl and pH 8, 1 mM EDTA) was performed

without incubation. The bead pellets were suspended in 20 µl of bead-fraction buffer. Bead-fraction buffer had the same composition as one part 10× binding buffer to nine parts DNA dilution buffer to avoid possible PCR bias based on buffer differences between the methylated and unmethylated fractions. Methylated fractions from FFPE samples

were stored as bead suspensions without eluting the DNA from the beads.

Methylated DNA from frozen tissue was eluted from the beads by a 10 min incubation at 65°C and 1000 rpm in a thermomixer. The beads were removed with a magnet. The eluted methylated DNA and unmethylated DNA fractions from frozen tissues were stored at -20°C. The fractions from FFPE samples were stored refrigerated for up to 3 months before CAPS.

For higher throughput, samples were fractionated in polypropylene round bottom microtiter plates with reduced wash buffer volumes. All bead washes were 150 µl. Sample mixing in microtiter plates was done at 850 rpm in a thermomixer.

Coupled Absorption PCR Signaling

CAPS Format I – MS-based detection

The amount of target DNA in the methylated and unmethylated fractions is determined by performing CAPS on each. PCR reactions for LC–MS detection were done in 20 µl volumes containing 1× Maxima Taq Hot Start Buffer, 2 mM MgCl₂, 0.8 mM dNTPs, 6% v/v DMSO, PCR primers at 0.5 µM each and 0.5 unit of hot start Maxima Taq (Fermentas, MD, USA). Unmethylated DNA fractions (2 µl) and suspended bead fractions containing methylated DNA (2.08 µl) were added to 18 µl of PCR master mix. The increased volume of the bead fraction compensated for the volume occupied by the suspended beads.

The cycling conditions were three cycles of 94°C for 2 min, 65°C (*MGMT* region I) or 67°C (*MGMT* region II) for 30 sec, 72°C for 30 sec followed by 28 cycles of 94°C for 30 sec, 65°C (*MGMT* region I) or 67°C (*MGMT* region II) for 30 sec and 72°C for 30 sec. A final incubation at 72°C for 5 min was followed by a holding step at 4°C.

Absorption master mix was composed of 148 mM HEPES pH 7.4, 148 mM KCl, 37 mM MgCl₂, 6 mM UpU (RiboMed, cat# I44), 6 mM GTP and 0.06 volume Abscriptase-I (RiboMed, cat# FP001). An Abscription master mix volume of 2 µl was combined with 10 µl of PCR reaction. Beads were removed from the PCR tubes before addition of the Abscription master mix using a magnetic rack designed for PCR tubes (RiboMed, cat# MR22-01). Abscription was done for 30 min at 76.4°C. Abscription reactions were stopped by reducing the incubation temperature to 25°C. Samples were prepared by mixing 5 µl of Abscription reaction with 20 µl of water in a 384-well plate. Ten microliters of diluted samples were analyzed by LC–MS.

LC–MS readout was performed on a Waters LCT-Premier ESI-TOF spectrometer and 2795 HPLC with a 3 µm, 2.1 mm × 20 mm Atlantis C18 column. Solvent A was HPLC grade water, 0.01% formic acid.

Solvent B was HPLC grade acetonitrile, 0.01% formic acid. The separation was performed over 8 min with a linear gradient of 0–21.6% Solvent B. An *m/z* range of 200–1000 was scanned. Abscript peaks were extracted from the total ion current data using singly charged and doubly charged *m/z* values of 446.8 (doubly charged uridylyl[3′-5′]uridylyl[3′-5′]guanosine [UUG]) and 894.2 (singly charged UUG). Signal intensities were the sums of the signals from singly charged and doubly charged ions represented as the areas under the chromatographic peaks.

CAPS Format II – fluorescent beacon-based detection

CAPS Format II is used to tag amplicons with APCs that encode 11-mer abscripts. When working with FFPE DNA or fewer than 30 copies of DNA from other sources, the methylated DNA is not eluted from the magnetic beads. DNA was measured by amplifying 1 µl samples of the unmethylated fraction and 1.08 µl of the suspended, bead-containing methylated fractions in 10 µl PCR reactions. Both the unmethylated and methylated DNA inputs from frozen tissues were 1 µl. The difference in the sample input volumes for FFPE DNA takes into account the volume occupied by the magnetic beads in the methylated fraction. The PCR master mix contained 0.25 units of Maxima Taq (ThermoFisher, MD, USA), 1× Maxima Hot start buffer, 0.8 mM dNTPs, 1.75 mM MgCl₂, 5.26% v/v DMSO and 1× target specific primers. Additional MgCl₂ was contributed by the DNA sample to give a final concentration of 2 mM. Additional DMSO was contributed by the primers to give a final concentration of 6% v/v. DMSO inclusion is essential for maximal amplification efficiency of heavily methylated fragments [31].

The PCR cycling conditions for *MGMT*-1 were: step 1, 95°C, 2.25 min; step 2, 57°C, 3 min; step 3, 72°C, 30 s; step 4, repeat steps 1–3, 2×; step 5, 88.9°C, 5 s; step 6, 57.7°C, 15 s; step 7, 72°C, 15 s; step 8, repeat steps 5–7 25× for 29 cycles or 27× for 31 cycles. Amplifiable DNA inputs to PCR reactions above 100 genomic copies were amplified for 29 cycles. Amplifiable DNA inputs to PCR reactions of 15–100 genomic copies were amplified for 31 cycles. All CAPS Format-II reactions used 50-fold concentrated target-specific/universal primer sets from Figure 3. Primers were made by Integrated DNA Technologies (IA, USA). The 50× primer mixes contained universal primers at 25 µM each. Target-specific primer concentrations ranged from 2.5 to 7.5 µM based on optimizations to maximize signal and minimize background. DMSO was included in 50× primer stocks at 37% v/v to maximize shelf life. The optimal PCR endpoints in Table 1 apply to all

Table 1. Endpoint PCR cycles versus DNA input.	
End point cycle number	CAPS DNA input
31	≤0.5 ng
29	0.5–2 ng
27	2–10 ng
25	10–50 ng
CAPS: Coupled Abscription PCR Signaling.	

the primer sets. Optimal PCR Phase 1 annealing temperatures are: *MGMT-1*, 57°C; *MGMT-2*, 65°C; *HFE*, 61°C; *MAL*, 57°C; and *SOWAHA*, 67°C.

After PCR cycling of FFPE DNAs, the magnetic beads were removed using a magnetic rack designed for PCR tubes (RiboMed, cat# MR22-01). The PCR reactions were combined with 10 µl of 1× Abscription mix containing 20 mM pApU (RiboMed, cat# IP14), GTP, CTP, UTP (8 mM each), dATP, dGTP (8 mM each), 1× Abscriptase-II (RiboMed, cat# FP-002), 1× CAPS-DNA polymerase (RiboMed, cat# FP-003) and 1 µM FAM-BHQ1-labeled molecular beacon (Ribomed cat# MB001). The Abscription reactions need not be carried out in a thermocycler. The Abscription phase is isothermal. However, it is convenient to carry out the entire reaction in a qPCR machine. Samples were incubated in an ABI 7000 Prism qPCR cycle for 15 repetitions of 40°C for 6 s and 41°C for 50 s. Each cycle was 1 min in duration when factoring in the 4 s ramp time. Temperature cycling during the Abscription phase is not required and was only performed because of programming requirements on the machine.

The rate of abscrip-dependent fluorescence increase is directly proportional to the amount of amplifiable target DNA in the CAPS reaction. For each batch of samples plus a no-DNA control, a component file containing the FAM fluorescence measurements in row orientation was exported from the ABI 7000 to Microsoft Excel where the slopes of the FAM fluorescence increase verses Abscription time were calculated. Cycle number or minutes of Abscription can be used interchangeably because each cycle is 1-min long. First a row of Abscription minutes (cycle numbers) was set up in the Excel worksheet corresponding to all of the fluorescence readings. The fluorescence increase rate was calculated using the Excel formula: SLOPE (FAM readings, Abscription minutes). The FAM and time ranges in the slope formula can be set to an arbitrary subset of the data to cover the linear portion of the relationship. The FAM increase rate for the no DNA control was subtracted from each of the sample FAM increase rates.

It was useful to plot the FAM increase verses Abscription time in order to confirm that the FAM increase was linear and to determine the range of the

linearity. FAM values for all samples were normalized to a time = 1 min value of 100. Graphs of FAM increase verses Abscription time were plotted using the Excel scatter plot option choosing the entire Abscription time range for the X-values and the entire FAM reading range for the Y-values (Figure 2B).

The linear relationship between the FAM increase rate and the DNA PCR input was determined with a titration of HeLa DNA that was processed by CAPS in parallel with the samples. The FAM increase rates for the calibrators were plotted using the Excel scatter plot option with FAM increase rates as the x-values and the calibrator DNA amounts as the y-values (Figure 2C). The experimental sample DNA amounts (y) were calculated from the sample rates of FAM increase (x), the slope (m) and intercept (b) of the calibration curve ($y = mx + b$).

The percent methylation was calculated as $(Me/Me+U) \times 100$, where Me is the amount of target in the methylated fraction and U is the amount of target in the unmethylated fraction.

MethylMeter with 30 genomic copies or less

The accuracy of DNA fractionation by the MethylMagnet magnetic beads at extremely low DNA inputs was demonstrated with reduced binding reaction volume and reduced bead input. Stock MBD-magnetic beads were diluted 100-fold into 1× binding buffer and 10 µl were distributed to 0.5 ml microcentrifuge tubes. The beads were pulled down with a magnet and the binding buffer was discarded. The beads were suspended in 2 µl of DNA (20–100 pg) that had been diluted into 1× binding buffer. The samples consisted of undamaged cell-pellet DNA from saliva, HeLa DNA and artificially methylated HeLa DNA. The DNA binding mixtures were incubated at 22°C, 1000 rpm for 1 h. At the completion of the binding reaction, the beads were pulled down with a magnet and the 2 µl supernatant fractions containing the unmethylated DNAs were transferred to PCR tubes. The beads were washed with 18 µl of complete PCR master mix (including Taq enzyme) and the wash buffer (PCR master mix) was added to each unmethylated DNA fraction to create a complete PCR reaction mix. The beads which con-

tained the methylated DNA fraction were suspended in 18 µl of complete PCR master mix and 2 µl of bead-fraction buffer. The methylated DNAs were amplified directly from the beads, similarly to the treatment of FFPE-methylated fractions. Both fractions were processed by CAPS Format II as described below, with the exceptions that end point PCR was done for 36 cycles and the Abscription reaction volume was 40 µl.

Primer design & validation

CAPS primer design involves first identifying target-specific primer sequences and then screening *in silico* for primer dimer potential after appending the 5' segment sequences (Figure 3). The target-specific primer sequences are obtained using Primer-Blast from the US National Center for Biotechnology Information web site [32]. The PCR product size options should be set to 40 bp minimum and 200 bp maximum to match the amplifiable DNA size range expected when working with FFPE or cell-free DNA.

A total of four primers are used in the fluorescence detection assay. The APC producing the 11-mer is larger than the APC for the 3-mer and is most efficiently constructed in a two phase PCR with four primers. In Phase 1, the forward primer has a truncated APC downstream (APC-DN) segment appended to its 5' end. The reverse primer has a universal reverse segment linked to its 5' end. These primers are used to create a tagged amplicon in Phase 1. A third and fourth primer, called the APC completion primer and the universal reverse primer, are responsible for amplification in Phase 2.

A text editor is used to paste the APC-DN segment sequence to the 5' end of one of the target primers. The sequence of the universal reverse segment is pasted to the 5' end of the reverse primer. Each composite primer sequence is tested in conventional primer development software (e.g., Oligo Analyzer® program, IDT, IA, USA [33]) for the potential to form self-dimers. Potential dimers with an annealed 3' end >3 nt are rejected. Forward and reverse composite primers that pass the self-dimer test are further tested for formation of heterodimers. Potential heterodimers with an annealed 3' end longer than 3 nt are rejected.

Candidate primer pairs are subjected to a Phase 1 annealing temperature gradient PCR followed by Phase 2 amplification with the APC completion primer and the universal reverse primer. DNA samples of 150 genomic copies per PCR are amplified in parallel with no-DNA controls for 30 PCR cycles. The completed reactions are subjected to Abscription for 15 cycles. Primers that produce sufficiently intense fluorescence signal and low background are accepted and assigned an optimal Phase 1 annealing temperature.

Only two primers are needed for the LC-MS-based detection. The APC primer encoding a trinucleotide abscrypt is short enough to order as a complete APC primer. Sample amplifications are done with a promoter target-specific primer and a target-specific reverse primer with no additional tags. Text versions of the APC primer and reverse primer are screened for primer dimer potential and then are tested for background level and signal intensity in an annealing temperature gradient experiment.

Detection of the *IDH1* R132H mutation by CAPS

AluI-digested FFPE DNA was subjected to CAPS Format II using *IDH1* R132H and wild-type primers as described above. The target-specific forward primer for the R132H allele (5' GGTAACCTATCATCATAGGTCA) was designed so that the R132H nucleotide (A) at 3' end of the primer is complementary to the DNA template corresponding to the R132H mutation. The R132H-specific primer forms a 3' terminal A:C mismatch with the wild-type R132 complement. The A:C mismatch is sufficient to block amplification of the wild-type target. The forward target-specific primer for the wild-type allele R132 (5' GGTAACCTATCATCATTTGGTCG) forms a 3' terminal G:T mismatch with the R132H allele. The G to T substitution 6 nt from the 3' terminus in the wild-type primer increases discrimination against the R132H allele. The reverse target-specific primer (5' TGCAAAATCACATTATTGCCAAC) is used with both forward primers.

The PCR cycling conditions for the *IDH1* allele amplifications were the same as for the methylation assays except that the Phase 1 annealing temperatures for the R132H primers and WT R132 primers were 60.7 and 58.5°C, respectively.

Methylation cutoffs

Methylation cutoffs for *MGMT* region-I and *MGMT* region-II were assigned using ten normal brain samples and nine primary glioma samples from the Austrian Institute of Technology (AIT). Cut-off values were set to maximize *MGMT* methylation assignment agreements with MSRE data. DNA methylation cutoffs for *HFE*, *MAL* and *SOWAHA* were assigned using 19 glioblastomas grades 2–4 with known CIMP status. Cut-off values were set to maximize agreement with G-CIMP assignments based on MethyLight analysis.

RNA sequencing for *MGMT* mRNA expression levels

Tumor pieces were separated from frozen bulk tumor with a scalpel on dry ice. Total RNA was isolated either using Maxwell 16 Total RNA Purification Kit

(Promega, WI, USA) or via sequential purification with SurePrep RNA/DNA/Protein Purification Kit (Fisher Bioreagents, PA, USA), followed by DNase treatment and further purification via mRNeasy kit (Qiagen, CA, USA). RNA was quantified on the Nanodrop 8000 (Thermo Fisher, MA, USA). RNA integrity was determined using TapeStation (Agilent, CA, USA) following manufacturer's recommendations.

100–200 ng total RNA was depleted of ribosomal RNA (New England Biolabs, MA, USA) and converted into barcoded sequencing libraries using TruSeq Stranded mRNA LT (Illumina, CA, USA). Libraries were sequenced on an Illumina HiSeq machine generating 3×10^7 100–130 base single-end reads per sample.

Alignment and postalignment processing were performed on an Amazon AWS instance running an Ubuntu 14 Linux environment. Raw sequencing results in the form of FASTQ files were used as input for alignment of the sequencing data to the human ENSEMBL reference RNA database GRCh37. ENSEMBL and human genome (hg19) annotation files were retrieved from the Tophat website, which links to the Illumina iGenome collection. Alignments were performed with Tophat2, which produced BAM alignment files to the human genome (hg19) [34]. Tophat BAM files were used directly as input into Cuffdiff2 to generate normalized RPKM values and differential expression analyses for *MGMT* mRNA expression determination. *MGMT* mRNA expression in tumors was compared with normal human brain and fold change in expression assigned.

Statistical analysis

Spearman's correlation coefficients and associated p-values were calculated in the R program using the `cor.test()` function in the stats package (R version 3.2.3 [35]). The Pearson product-moment coefficient was calculated to analyze the LC–MS and molecular beacon detection data.

Results

Workflow

Figure 1 outlines the bisulfite-free, MethylMeter DNA methylation assay. The method relies on the separation of methylated DNA from unmethylated fragments with the use of magnetic beads bearing a GST-MBD2 fusion protein. DNA samples are first fragmented with a restriction endonuclease that is unaffected by methylation, usually by AluI restriction, to isolate a targeted segment of the CpG island from flanking regions that might bias the fractionation of unmethylated targets. The extent of AluI cleavage is verified by amplifying AluI digested samples and uncut controls using an arbitrary detection primer pair for the *HFE* CpG

island. This target is sensitive to AluI cleavage due to an intervening AluI site. Due to the high frequency of AluI sites flanking the target, incomplete digests of the *MGMT* CpG island are adequate to unlink neighboring CpG islands based on the identification of unmethylated targets from samples with AluI cleavage frequencies as low as 67%.

For analysis of the *IDH1* R132H mutation, an aliquot can be removed prior to fragmentation and fractionation with MethylMagnet or can be run on the DNA in the unmethylated fraction, where the fragment containing the mutation partitions. DNA fragmentation is relevant only for the DNA methylation assay. Mutation detection by CAPS will give consistent results regardless of linkage to neighboring sequence.

Fragmented target DNAs are separated into methylated and unmethylated fractions using MethylMagnet [17], a magnetic bead bound GST-MBD2-Me-CpG binding domain protein (Figure 1A, step 1). Methylated target DNA binds to the magnetic beads, while unmethylated targets remain in the supernatant fraction. The amount of target DNA in each MethylMagnet fraction is measured and used to calculate the percentage methylation for that target.

The amount of target DNA in each fraction is determined using a combination of end point PCR and Abscription or CAPS. The targeted CpG-island segment is amplified using primers to attach an APC to each amplicon. The promoter is activated in the course of copying the target (Figure 1A, step 2a). Signal molecules in the form of short, abortive RNA transcripts are generated after the PCR step by adding RNA precursors and an APC-specific RNA polymerase referred to as Abscriptase® (Figure 1A, step 2b).

Two formats for the CAPS assay have been developed, each with its strengths and weaknesses. In Format I, trinucleotide abscrips are generated and then quantified by LC–MS (Figure 1B, steps 2a & 3a). In Format II, 11-mer abscrips are generated and quantified by measuring fluorescence from a molecular beacon (Figure 1B, steps 2a & 3a). The 11-mer abscrips anneal to a single-stranded segment of the loop in the molecular beacon (Figure 2A, step 2). Extension of the annealed abscrip by a DNA polymerase (CAPS DNA polymerase) opens the beacon (step 3) and separates a fluorescein at the beacon 5'-end from a BHQ-1 quencher at the beacon 3'-end [36]. The rate of fluorescence increases during the Abscription reaction is directly proportional to the number of input DNA molecules sampled in the PCR reaction (Figures 2B & C).

Sensitivity of MethylMeter

We determined the sensitivity and accuracy of MethylMeter assay with DNA samples containing

Figure 4. MethylMeter accuracy at low DNA inputs. Low DNA-input samples were processed with the MethylMagnet kit followed by Coupled Absorption PCR Signaling detection. DNA MethylMagnet inputs were bound in 2 μ l reactions containing 0.1 μ l of beads. The entire unmethylated and methylated fractions were each measured in a single Coupled Absorption PCR Signaling reaction to minimize sampling error for the methylation determination. (A–D) Show fluorescence increases versus Absorption time for samples containing six genomic copies (A, B & C) and 15 genomic copies (D). The *HFE* gene was tested to compare assay performance for a completely unmethylated target (normal DNA, A) versus a completely methylated target (artificially methylated HeLa DNA, B). The low bead input showed complete binding of the methylated target and no nonspecific binding of the unmethylated target (E, rows 1 and 2). (C & D) Show the FAM increase data for the imprinted *SNRPN* gene at inputs of 6 and 15 genomic copies. The methylation percentages for inputs from 6 to 30 genomic copies were close to the expected level of 50% (E, rows 4–6).

FAM: Fluorescein fluorescence increase; Me: Methylated fraction; U: Unmethylated fraction.

between 6 and 30 genomic copies using small-scale bead inputs (0.1 μ l) and binding reaction volumes (2 μ l). Fractionation of six copies of unmethylated *HFE* gene from normal (unmethylated) saliva DNA showed no evidence of nonspecific binding while six copies of

the *HFE* gene from artificially methylated HeLa DNA showed complete *HFE* methylation (Figure 4A & B). The fractionation of five HeLa replicates was highly consistent with an average DNA methylation level of 98.7% ± 2.7.

The sampling error expected when distributing six genomic copies to the MethylMagnet binding reaction was reflected in the variability of the total amounts of DNA among the samples but not in the methylation results, where the fractions are homogeneous with respect to methylation (Figure 4E, rows 1 and 2 vs row 3). The fractionation of the imprinted SNRPN gene at 6, 15 or 30 genomic copies of HeLa DNA was reasonably close to the 50% methylation level expected for an imprinted gene given the potential for sampling error to skew MethylMagnet inputs that are mixtures of methylated and unmethylated fragments (Figure 4E, rows 4–6).

Coupled Abscription PCR Signaling

The copy number of each target in the bead (methylated) and supernatant fractions (unmethylated) are measured using a combination of end-point PCR, followed by real-time fluorescence detection of amplicons by Abscription. The PCR phase generates amplicons that are each tagged with a specific APC. The APCs used here reiteratively generated either a 3-mer or an 11-mer abscript.

A full length APC for the production of a trinucleotide abscript is linked to a target-specific primer and used with a conventional reverse target-specific primer to tag amplicons. The single-stranded version of the APC is inactive for Abscription but becomes activated when a target is copied. The first three PCR cycles use an extended denaturation time to compensate for the slow kinetics of denaturation of fully methylated DNAs [37].

The APC for production of an 11-mer is too long to merge full-length with a target-specific primer. In this case, a truncated downstream APC segment is linked to a target-specific primer (Figure 3, sequences A and B). The incomplete APCs on the initial amplicons are made full-length with an APC-completion primer (Figure 3, sequence E).

The PCR program for the 11-mer encoding APC is broken into two phases that correspond to the different steps in APC assembly. Phase 1 consists of the first three cycles, which are used to establish an amplicon with the truncated APC. This phase incorporates a 2.25 min denaturation time to accommodate the slow denaturation of fully methylated target. Phase 1 conditions are optimal for the APC target-specific (forward) primer and a reverse target-specific primer with a 5' universal sequence (Figure 3, sequences C and D). In Phase 2, an unmethylated copy of the target sequence exists and subsequent PCR cycles use a 5-sec denaturation time. Phase 2 conditions favor a second set of primers present at 0.5 μ M (Figure 3, sequences E and F). The APC completion primer targets the truncated APC sequence on the amplicon and produces a complete

active promoter (Figure 3, Phase 2, sequence E). The universal reverse primer is designed to anneal under the same conditions as the APC completion primer and uncouples Phase 2 amplification conditions from those of the reverse target-specific primer (Figure 3, Phase 2, sequence F). Phase 2 conditions are designed to maximize the production of amplicons with full-length promoters at the expense of amplicons with the incomplete downstream promoter.

As the PCR and Abscription steps are performed sequentially, it is important to choose a PCR endpoint that does not produce enough amplicons to saturate the downstream Abscription reaction. Table 1 gives guidelines for the appropriate number of PCR cycles for *MGMT*, *HFE*, *MAL* and *SOWAHA* detection at different DNA inputs into the CAPS reaction. Input is based on amplifiable DNA. The amount of amplifiable DNA in a sample is known before the DNA fractionation step because the AluI digests are calibrated in terms of amplifiable genomic copies/ μ l during the evaluation of AluI cleavage (Methods and materials, Copy number and Amplifiable DNA determination). It is especially important to determine the amplifiable DNA content when processing FFPE DNA because formalin fixed DNA is heavily damaged and the total DNA concentration is usually significantly higher than the amplifiable fraction.

MGMT promoter methylation

CAPS-based detection is more sensitive than other PCR-based methods in part because each amplicon produces multiple signals. CAPS with LC–MS detection of trinucleotides is 100–1000-fold more sensitive than comparable qPCR on undamaged DNA samples [17]. The enhanced sensitivity should make CAPS detection highly effective with heavily damaged formalin-fixed DNA samples. We tested MethylMeter with fluorescent detection on a set of glioma samples that had been split and then fixed with formalin (FFPE) or RCL2 [30]. FFPE samples are prone to analytical failure because the fixation process introduces DNA damage in the form of crosslinks and strand breaks. RCL2 fixation is expected to have a higher success rate because much less DNA damage is induced upon fixation [38,39]. The previous *MGMT* promoter methylation analysis of the FFPE fixed sample set by MSRE-qPCR failed at AIT but the RCL2 samples were successfully analyzed [30]. To test the sensitivity of the MethylMeter molecular beacon assay, we analyzed the AIT FFPE samples and evaluated the accuracy with the available MSRE-qPCR data from the RCL2 samples.

The *MGMT* CpG island is broken into two targets; Region I is defined by AluI cleavage sites situated at -109 and +149 from the transcription start site (TSS)

and Region II is defined by sites at -313 and -109. FFPE DNAs from gliomas were purified and processed as described in Materials & methods. All results were calibrated and expressed as genomic copies/ μ l in the methylated and unmethylated fractions using titrations of HeLa DNA processed in CAPS reactions run in parallel with the MethylMagnet fractions. Figure 5 shows comparisons of molecular beacon-based fluorescence detection (Format II) to LC-MS based detection (Format I) [17]. Methylation measurements were

targeted to the downstream segment of the *MGMT* CpG island that includes a segment that had previously been analyzed by MSRE-qPCR on RCL2-fixed samples (Region I, Figure 5A) [30]. The methylation cutoff for the MSRE-qPCR detection was set at two standard deviations above the average methylation level of normal brain samples [30]. A cutoff of 10% for the MethylMeter LC-MS and molecular beacon detection maximized the agreement among these methods and MSRE.

Figure 5. Molecular beacon-based detection versus LC-MS detection and methylation sensitive restriction endonuclease. (A) is a map of the *MGMT* CpG island and two target fragments generated by *Alul* cleavage (Regions I and II). Methylation of either Region I (37 CpGs) or II (28 CpGs) is sufficient to reduce *MGMT* expression. The gray segment in Region I (nine restriction sites) was analyzed by MSRE-qPCR. Only Region I was analyzed by MethylMeter in (B). Percent methylation scores are shown for molecular beacon detection versus LC-MS detection and MSRE. Methylation assignments are shown for the Epityper method. The molecular beacon data was generated 3 years after the LC-MS experiment on the same DNA samples in B. The molecular beacon and MSRE results were discordant for samples 8, 19, 21 and 31. The MethylMeter results in these cases were consistent with survival data. The discordant result between the molecular beacon and LC-MS assignments for sample 15 is resolved in favor of the molecular beacon method based on survival. (C) shows a comparison of the MethylMeter fluorescence assay data for recurrent tumors for Regions I and II. *MGMT* mRNA data available for three discordant methylation assignments are consistent with the MethylMeter results. Statistical analysis comparing the results for the different methods to each other, survival and mRNA data is given in Table 2. LC-MS: Liquid chromatography mass spectrometry; Me: Methylated fraction; MSRE-qPCR: Methylation sensitive restriction endonuclease quantitative PCR; U: Unmethylated fraction.

Overall there was good agreement between the CAPS LC–MS and molecular beacon methods considering they were performed 3 years apart with the same DNA samples (Figure 5B & Table 2). The single discordant result between the molecular beacon and the LC–MS detection for sample 15 was resolved in favor of the molecular beacon detection based on survival data. In cases of discordant methylation assignments, the methylation assignment that was not clearly inconsistent with the ranking of the linked survival data was regarded as correct. A comparison of the MethylMeter beacon results against the MSRE results shows four discordant results (Figure 5B). The apparent disagreement over sample 8 might not be real given that it is close to the cutoffs for both methods. Survival data for samples 19, 21 and 31 are more consistent with the MethylMeter data.

The same set of RCL2-fixed samples were analyzed by AIT using the bisulfite and MS-based EpiTyper method [40]. The region analyzed by EpiTyper overlapped with Region I and II but did not overlap with the region analyzed by MSRE (Figure 5A). There were only two discordant results between the EpiTyper and MethylMeter assignments. The survival data for sample 31 were more consistent with the MethylMeter assignment. There were no survival data available for sample 8 to resolve the disagreement.

Pearson product moment correlation coefficient and p-value for the molecular beacon method versus LC–MS detection showed as expected that these detection methods are highly correlated ($r = 0.95$; $p = 2.3 \times 10^{-7}$). Spearman's correlation coefficient showed the molecular beacon CAPS method was moderately correlated with MSRE ($\rho = 0.49$; $p = 0.043$). The molecular beacon method was highly correlated with survival ($\rho = 0.85$; $p = 0.0041$) in the AIT primary tumor samples versus a correlation of 0.61 ($p = 0.023$) between MSRE and survival.

A second *MGMT* validation was performed focusing on a set of 26 recurrent high-grade GBM tumors from frozen tumor tissue for which mRNA expression data were also obtained [41]. These samples were previously analyzed for *MGMT* methylation by undisclosed methods at multiple clinical study centers. The mRNA abundance data were used to resolve discordant results with the clinical site methylation assays. DNA methylation levels were measured for *MGMT* CpG island Regions I and II. Methylation of either of these regions can reduce *MGMT* expression [42–44]. Our methylation assignments were always consistent between the two regions suggesting that either region could be used to analyze recurrent tumors (Figure 5C & Table 2). Comparison of the MethylMeter results to the methylation assignments from the clinical sites showed four disagreements. In the three discordant cases where *MGMT* mRNA data were available, the RNA levels were consistent with the MethylMeter methylation assignments (Figure 5C, samples 40, 44 and 58).

There were two cases (samples 39 and 57) where an unmethylated call by MethylMeter was matched with a low RNA level. This kind of result is not unexpected because an unmethylated status does not guarantee production of a mature mRNA. There were two cases (samples 40 and 58) where the clinical sites assigned a methylated status to a sample that showed higher or equal levels of *MGMT* mRNA expression compared with normal. This kind of result implies that the clinical site methylation assignment was incorrect. The molecular beacon methylation data rankings for Regions I and II showed the expected negative correlations with *MGMT* mRNA level (Table 2).

GliomaSTRAT & IDH1 mutation detection

The high sensitivity of the CAPS-based detection and the nondestructive nature of MethylMeter maximize the number of genes that can be analyzed from a given

Table 2. Correlations among methods, survival and mRNA levels for <i>MGMT</i> promoter.			
Comparison		R†	p-value
MethylMeter Beacon Region I	MethylMeter MS Region I	0.95 p‡	2.2 × 10 ⁻⁷
MethylMeter Beacon Region I	MSRE Region I	0.49	0.043
MethylMeter Beacon Region I	Survival	0.85	4.1 × 10 ⁻³
MethylMeter MS Region I	Survival	0.78	2.2 × 10 ⁻³
MSRE Region I	Survival	0.61	0.023
MethylMeter Beacon Region I	MethylMeter Beacon Region II	0.83	8.5 × 10 ⁻⁸
MethylMeter Beacon Region I	mRNA	-0.57	3.6 × 10 ⁻³
MethylMeter Beacon Region II	mRNA	-0.63	1.0 × 10 ⁻³
†Pearson product moment coefficient.			
‡Spearman's correlation coefficient.			
MS: Mass spectrometry.

Figure 6. GliomaSTRAT results for low-grade and high-grade gliomas. FFPE samples were separated into methylated and unmethylated fractions followed by CAPS reactions to measure the CIMP markers *HFE*, *MAL*, *SOWAHA* and the drug resistance marker *MGMT*. The maps show the locations of the targeted AluI fragments (unfilled rectangles), the MethylMeter PCR targets (black rectangles) and the MethylLight targets (gray triangles) from Noushmeir *et al.* [27]. The double arrow in *HFE* represents the CAPS target for validating AluI cleavage. CAPS reactions for the *IDH1* R132H mutation were performed with a promoter-primer forming a mismatch at the site of the mutation. The results labeled grade 2 indicate a CIMP-plus low-grade glioma that is predicted to be responsive to temozolomide. The sample labeled GBM is predicted to be a high-grade glioma based on the CIMP-minus and *IDH1* R132H-minus results. The unmethylated status of *MGMT* predicts unresponsiveness to temozolomide. The molecular results were consistent with the pathologists' diagnoses. CAPS: Coupled Absorption PCR Signaling; CIMP: CpG island methylator phenotype; FFPE: Formalin fixed and paraffin embedded; GBM: Glioblastoma multiforme; WT: Wild-type.

sample. MethylMagnet fractions potentially can be used for other types of analysis because the DNA is not modified by the assay. We demonstrated this by analyzing glioma samples for the CpG Island Methylator Phenotype and the *IDH1* R132H mutation that is often used as an alternative marker to infer this phenotype in the absence of methylation data [27,28].

DNA methylation analysis of gliomas demonstrated that a G-CIMP exists that can identify patients with improved prognosis within histological grades 2, 3 and 4 [27]. In the majority of cases, the methylated G-CIMP is caused by gain of function mutations in the *IDH1* gene, which results in remodeling of the epigenome and production of G-CIMP-associated changes in transcription [28]. We reduced the original eight-gene G-CIMP methylation signature to three genes (*HFE*, *MAL* and *SOWAHA*) using MethylMeter with LC-MS detection (data not shown). The CIMP targets were defined by AluI cleavage sites located at the following coordinates relative to the TSS: -46 to +146 (*HFE*); +154 to +298 (*MAL*) and -1046 to -455 (*SOWAHA*). Figure 6 shows the result of the three-gene test applied to tumor samples diagnosed as WHO

grade 2 (low grade) or grade 4 glioblastoma (high grade). A methylated call for at least two of the three CIMP CpG islands caused a G-CIMP-plus assignment. The G-CIMP results were concordant with the *IDH1* R132H results as expected and the combined methylation and mutation results agreed with the clinical diagnoses.

Caveats

To maximize the accuracy of the method for specific gene targets, two precautions should be taken involving the DNA fractionation step. First, DNA fragmentation should be verified before the binding step to ensure that the target is unlinked from neighboring CpG sites. Site-specific cleavage by a restriction enzyme can be tested with PCR primers that are sensitive to cleavage at a surrogate site. Second, different targets are expected to compete for binding at different efficiencies at saturating DNA inputs. This problem can be avoided by performing a bead volume titration against the largest anticipated DNA input. The most stringent approach is to use an artificially methylated test DNA.

Conclusion & future perspective

A major advantage of the MethylMeter assay is its sensitivity and ability to detect even small DNA targets (≥ 40 bp) in the size range typical of DNA found in many clinical samples, including FFPE, blood and urine. The affinity approach avoids chemical damage to the sample that occurs with bisulfite-based methods. When used in the correct proportion, the GST-MBD2 magnetic beads are able to accurately fractionate DNA inputs at least as low as 20 pg (six copies). Larger inputs (>1 μ g) can be fractionated with larger bead volumes and by further decreasing the number of PCR cycles during CAPS. The addition of the Abscription-based linear signal amplification to the PCR target amplification increases the sensitivity of the assay beyond that achieved with qPCR [17]. Consequently, a high success rate with FFPE samples is possible without preamplification or nested PCR. Unlike in qPCR, every amplicon generates multiple signals. qPCR has the advantage of a one-tube homogeneous format. We have not had to take precautions against cross contamination among PCR reactions during the Abscription setup because the Abscription step produces linear signal amplification. The development of a homogeneous CAPS assay is in progress. As a novel method, MethylMeter would benefit greatly from validation by other investigators and standardization among multiple laboratories.

The MethylMeter method appears to be highly accurate, based on its consistency with survival data and *MGMT* mRNA levels in cases where it is discordant with other methylation detection methods. Affinity methods have the advantage that they are less sensitive to methylation pattern heterogeneity than methods that target a few specific CpG sites. For example, the presence of one or more unmethylated CpG sites in a targeted site of an otherwise methylated DNA can lead to an incorrect inference of the overall methylation status for methods based on MS-PCR or MSRE [45]. This situation should not affect affinity-based detection because every methylated CpG site can contribute to a binding event. Our measurement of methylation of the imprinted SNRPN CpG island yields the expected 50%, despite the heterogeneous distribution of specific CpG site methylation demonstrated by bisulfite sequencing [17]. Compared with enrichment methods, measurement of target copy number in both the methylated and unmethylated fractions and calculation of the percentage of the targets that are methylated internally control for potential target copy number changes.

New MethylMeter assay development is simple compared with other methods because the discrimination between methylated and unmethylated DNAs is performed in a standardized binding reaction. Most gene targets can be analyzed using the same binding

conditions, although the stringency of the binding reaction can be changed by optimizing the NaCl concentration of Wash Buffer 2. Primer design in MethylMeter has fewer constraints than with bisulfite-based or MSRE methods. Target sequence complexity is not reduced by bisulfite treatment, so more potential priming sites are available. Target-specific primers can be placed at any arbitrary region in a target sequence. There is no requirement to include or exclude CpG sites. Forward and reverse primers can be placed arbitrarily close, as there are no probes to accommodate. This is an advantage in development of assays for heavily damaged sample DNAs or for cell-free DNA from bodily fluids. We have developed amplification targets as small as 40 nt. A further simplification is that the Abscription signal is independent of the target sequence, so Abscription will proceed with equal efficiency with any gene target that is tagged with the same APC. Development of new assays is limited to new primer design and determination of optimal Phase 1 annealing temperature. The later step documents the relative levels of signal to background. This process typically takes <1 week for an acceptable primer set.

The nondestructive nature of MethylMagnet allows the DNA in leftover methylated or unmethylated fractions to be used for other assays. This flexibility can be useful if sample sizes are severely constrained. The GliomaSTRAT assay exploits the high sensitivity of CAPS-based detection with heavily damaged DNA samples. DNA methylation analysis of four genes gives useful information on the potential response to temozolomide (*MGMT* methylation) and tumor grade based on G-CIMP (*HFE*, *MAL* and *SOWAHA* methylation). The G-CIMP assignment is confirmed by detection of the *IDH1* R132H mutation. An objective molecular assessment of tumor grade is an important complement to histopathology which is subject to a high level of interobserver discordance [46,47]. Accurate classification of grade 2 gliomas is important because treatment with temozolomide can induce hypermutation, driving them to aggressive grade 4 tumors [48,49].

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Executive summary

Aim

- Development of a DNA methylation profiling technology that is bisulfite-free, sensitive, quantitative and suitable for use with formalin fixed and paraffin embedded (FFPE) and other clinical samples containing fragmented DNA.

Materials & methods

- We developed a high sensitivity DNA methylation assay for stratifying gliomas by combining affinity-based separation of methylated and unmethylated DNA with target quantification in each fraction with Coupled Absorption PCR Signaling (CAPS). CAPS assays were developed for measuring *MGMT* methylation, which predicts Temozolomide response, determining CIMP using the methylation level of three other genes, and detection of the *IDH1* R132H mutation associated with CpG island methylator phenotype.
- Fluorescent detection was developed based on abscrip-dependent opening of a molecular beacon.

Results

- The MethylMeter method can accurately measure methylation with DNA inputs as small as six genomic copies.
- The molecular beacon read-out is consistent with LC-MS readout sensitivity and accuracy.
- The MethylMeter molecular beacon analysis of *MGMT* methylation is consistent with survival and mRNA expression data.
- CAPS can also be used for mutation detection during the DNA methylation profiling assay, without DNA sequencing.
- High sensitivity with FFPE DNA samples allowed analysis of four methylation targets and one mutation in the GliomaSTRAT assay. This information is clinically valuable in management of gliomas.

Conclusion

- MethylMeter assays are highly sensitive and well-suited to analyze FFPE samples.

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