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MethylMeter®: A Quantitative, Sensitive, and Bisulfite-Free Method for Analysis of DNA Methylation

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1. Introduction

In this chapter we present a new *bisulfite-free* method to detect and quantify DNA methylation and its application to the detection of imprinting disorders such as Prader-Willi (PWS) and Angelman (AS) syndromes. The method, called MethylMeter®, combines affinity separation of methylated and unmethylated DNA with CAP™ (Coupled Abscription®-PCR), a new quantitative and sensitive signal generation process. In order to validate MethylMeter®, we analyzed samples from 54 patients diagnosed with Prader-Willi or Angelman syndromes, as well as samples from normal patients. Results were compared to the results obtained previously on these samples using bisulfite-based TaqMan® Methylation-Specific PCR (MS-PCR). Methylation detection with CAP was as accurate as with TaqMan, but was approximately 2000 times more sensitive. Methylated DNA was separated from unmethylated DNA with the use of magnetic beads bearing a new methyl-CpG binding domain protein. The amount of the normally imprinted SNRPN promoter region present in the bound and unbound fractions was used to determine the relative amounts of methylated and unmethylated SNRPN promoter in the sample. The results were 100% concordant with previous results generated with MS-PCR, but significantly less patient DNA and time were required to obtain results, which are more quantitative than MS-PCR. CAP based detection can be accomplished without fluorescent probes and in fewer cycles than with other PCR methods. Because methylated DNA is detected based on purification of methylated DNA, rather than on chemical conversion of unmethylated DNA, the disadvantages of bisulfite treatment are avoided. DNA is not degraded allowing analysis of samples as small as 1 ng. CAP primer development is not limited by the effects of reduced sequence complexity or a requirement to overlap primers with CpG sites in the target DNA.

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1.1 Current methods for detecting DNA methylation in clinical samples

The most commonly used methods for analyzing the DNA methylation levels of Differentially Methylated Regions (DMRs), such as CpG islands, and individual CpG sites, utilize treatment of patient DNA with the chemical bisulfite. Bisulfite converts unmethylated cytosine (C) residues to deoxyuracil (dU), while leaving methylated cytosine (MeC) unchanged. Upon PCR amplification of bisulfite-treated DNA, the MeC is copied to C and dU is copied to thymine (T). As a result, the retention of cytosine at a specific position indicates methylation. The modified DNA can then be analyzed by methods that detect the sequence difference between the amplified DNA generated from the methylated and unmethylated, bisulfite-treated starting material.

In methylation-specific PCR (MSP), oligonucleotides that hybridize to a region on the DNA containing 1 or more potentially methylated CpG sites are added to the bisulfite-treated DNA. The oligonucleotides are designed to hybridize either to a DNA sequence corresponding to bisulfite-treated methylated DNA (still contains C), or to a sequence corresponding to bisulfite-treated unmethylated DNA (now contains dU), and PCR is performed in order to amplify the methylated and/or unmethylated target DNA (Herman, et al., 1996). Amplicons are analyzed by gel electrophoresis. Alternatively, fluorescence-based quantitative real-time PCR can be performed on bisulfite-modified DNA (Eads, et al., 2000; Zeschnigk, et al., 2004). Adaptations of quantitative real-time PCR utilize Taqman probes to generate a fluorescent signal or blocker oligonucleotides to prevent amplification of unmethylated DNA, resulting in increased assay sensitivity (Cottrell et al., 2004). Pyrosequencing is also utilized for methylation quantitation from bisulfite modified DNA (Tost et al., 2003).

An advantage of bisulfite modification is that it is a widely used and established procedure and allows analysis of methylation at single CpG sites in the DNA. Some of the disadvantages to this assay are that bisulfite treatment of DNA can destroy a large percentage of the input DNA, resulting in limited sensitivity and the requirement for large amounts of DNA. Quality control assessment of bisulfite treated DNA is necessary before performing a detection assay to avoid misleading results. Extensive degradation can introduce sampling errors when few molecules are long enough to be amplified (Ehrich et al., 2007). Bisulfite treatment also creates DNA targets that are now very A-T rich, having converted unmethylated C to T, and this complicates the design of specific probes for PCR amplification. Bisulfite treatment remains the main source of variability for methylation detection, particularly in samples containing already degraded DNA, such as Formalin Fixed Paraffin Embedded (FFPE) tumor tissues.

Other DNA methylation detection methods that do not use bisulfite rely on detection of methylated DNA by restriction enzyme analysis. The DNA is treated with either a MSRE (methylation-sensitive restriction enzyme) or a MDRE (methylation dependent restriction enzyme), amplified and then analyzed by microarray or gel analysis. MSREs are restriction enzymes, which cut at DNA only if the C in a CpG site is unmethylated. MDREs are restriction enzymes that require CpG methylation for cleavage. By treating DNA with either of these enzymes and subsequent comparison to a control sample, the methylation state of a DNA sample *at that site* can be determined. If digestion of a sample occurs after treatment with a MDRE, then the DNA is assumed to be methylated. Conversely, if the DNA is uncut

when treated with a MSRE, then this sample is also assumed to be methylated. By comparing the amount of cut vs. uncut DNA, the level of methylation can be estimated. A common read out for this type of methylation analysis is the subsequent amplification and fluorescent labeling of the digested DNA. The fragments can then be hybridized to a library microarray and analyzed (Lipmann et al., 2004) or simply resolved by electrophoresis. Quantitative real-time PCR is another mode of analysis (Ordway, et al., 2006). An advantage of MSRE/MDSE digestion is that no pretreatment of the DNA is necessary, although it is often performed in conjunction with bisulfite treatment of DNA in a procedure called COBRA (Xiong & Laird, 1997). Some disadvantages with this application are that it is a rather lengthy procedure and is dependent on the presence of specific restriction enzyme recognition sequences near the region of the target DNA.

A final method that is commonly employed is chromatin immunoprecipitation (ChIP) by the use of antibodies against methyl binding proteins. Typically, cells are fixed and then methylated DNA is immunoprecipitated by the use of antibodies specific for methyl binding proteins. The resulting DNA is amplified, labeled and analyzed by hybridization in a microarray assay. The advantages of this method are that the assay can be performed from live cells with little or no DNA purification required. The assay also has increased sensitivity, as unwanted and contaminant DNA are removed prior to analysis. However, the procedure is very time-consuming, involves several steps and requires expensive reagents. Some assays may take as long as five days to complete.

1.2 Imprinting disorder diagnosis by analysis of DNA methylation

Genetic alterations in DNA methylation that affect imprinting play important roles in Prader-Willi syndrome (PWS) and Angelman syndrome (AS). The imprinted genes in chromosome 15 region 15q11.2-q13 that are associated with PWS and AS, including the SNRPN (small nuclear ribonucleoprotein peptide N) promoter region, are normally methylated and unexpressed in the maternal chromosome and unmethylated and expressed in the paternal chromosome. Loss of the unmethylated and expressed paternal copy by deletion, maternal uniparental disomy (UDP) or by imprinting errors, leaving the methylated and unexpressed maternal copy as the only version of the gene, is associated with PWS due to loss of paternal expression. Conversely, AS is associated with the loss of the maternal copy of 15q11.2-q13 which can occur through deletion, mutation in the maternally expressed gene Ubiquitin-protein ligase E3A (UBE3A) or paternal UDP. Methylation analysis of the SNRPN promoter is used to confirm diagnosis of PWS although methylation studies alone do not define the genetic basis for the diagnosis. A positive result of a methylation analysis leads to follow-up studies to define the genetic cause.

The most commonly used diagnostic methylation tests for PWS and AS are MSP and Southern blot assays using methylation sensitive restriction enzymes (Glenn et al., 1996; Kubota et al., 1997; Ramsden et al., 2010). Methylation-specific multiplex ligation dependent amplification (MS-MLA) has also been used to identify the methylation status of the PWS region and to detect copy number changes in the region (Nygren et al., 2005). The Southern blot assay and one version of MS-MLA depend on the use of methylation sensitive restriction enzymes which probe the methylation status of one or more CpG sites. Results can be affected by incomplete digestion of genomic DNA or rare SNPs affecting restriction sites (Ramsden et al., 2010). MSP and alternative versions of MS-MLA use bisulfite treated DNA.

1.3 Abscription® – Based signal amplification

Abscription, short for Abortive Transcription, is a robust and isothermal signal amplification process that utilizes an RNA polymerase, called Abscriptase®, to generate thousands of specific short RNA oligonucleotides per minute from an artificial promoter called an Abortive Promoter Cassette (APC, Figure 1). Abscription exploits the natural phenomenon of abortive transcription during the initiation of transcription where RNA polymerases synthesizes large numbers of very short RNA molecules in the range of 2-12 nucleotides (Hsu et al., 2003; Hsu et al. 2006; Vo et al., 2003a; Vo et al., 2003b). Abortive transcription occurs very rapidly because RNA polymerase does not dissociate between rounds of short RNA synthesis and, if maintained in the abortive synthesis mode, will continue making short RNAs until nucleotide substrates are consumed. Each APC is designed to produce a short oligonucleotide of a different sequence and mass at turnovers of approximately 10,000 per minute. By attaching an APC to a biomarker target and measuring the amount of abscript produced from the APC, Abscription can be use to give a quantitative measurement of the amount of the target present. The "aborted" transcripts, or abscripts, can be quantified by several methods, including mass spectrometry (MS), capillary electrophoresis (CE) and rapid Thin Layer Chromatography (rTLC).
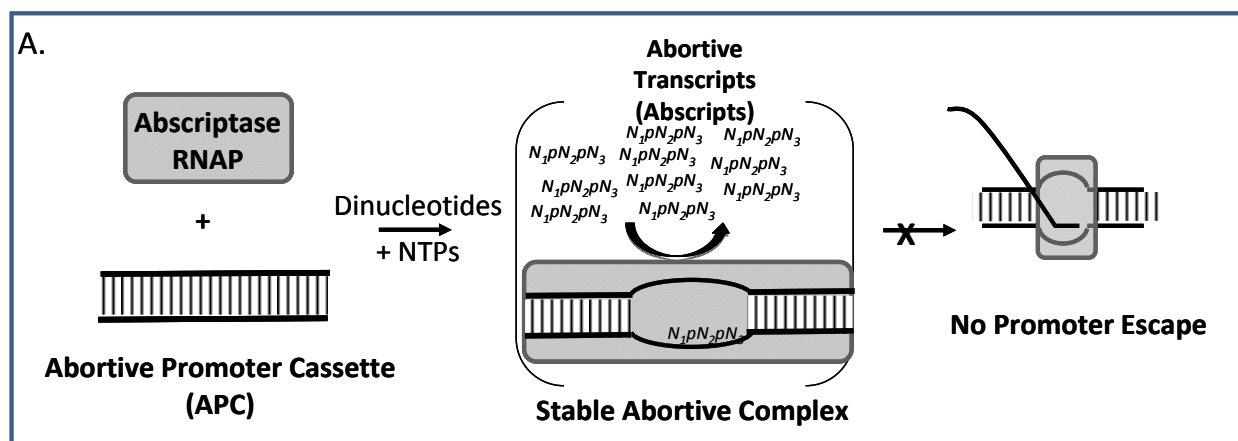

Fig. 1. Signal generation by Abscription® (Abortive transcription)

Abscription is catalyzed on a DNA template called an Abortive Promoter Cassette that encodes an RNA polymerase binding site and the start site for transcription. Abscripts are initiated with a dinucleotide complementary to the start site. Signal generation by Abscription exploits the natural phenomenon of abortive transcription which occurs during the initiation of transcription. Following promoter binding RNA polymerase temporarily synthesizes large numbers of RNA molecules in the range of 2-12 nucleotides. In normal transcription, the RNA polymerase undergoes a conformational change that allows promoter escape and entry into the processive elongation phase of the transcription cycle. Artificial promoters (APCs) and an abortive RNA polymerase, Abscriptase®, have been developed to trap the enzyme in the abortive phase and produce only abortive transcripts of specific sequence.

The highest turnovers are produced by APCs encoding trinucleotides and their synthesis from a dinucleotide initiator and a single NTP (Figure 2). For example, the trinucleotide

abscript GpApG (GAG) is synthesized reiteratively by Abscriptase by an APC directed joining of the dinucleotide GpA and GTP. The sequences of the promoter and the initially transcribed segment have significant effects on the lengths and rates of synthesis of abortive transcripts (Hsu et al., 2006). Optimization of these sequences allowed the development of APCs that efficiently produce a variety of trinucleotide abortive transcripts (Hanna, 2006, 2008, 2009). There are 64 different trinucleotides that can be made from the four standard nucleotides. APCs encoding over 20 of these trinucleotides have been developed.
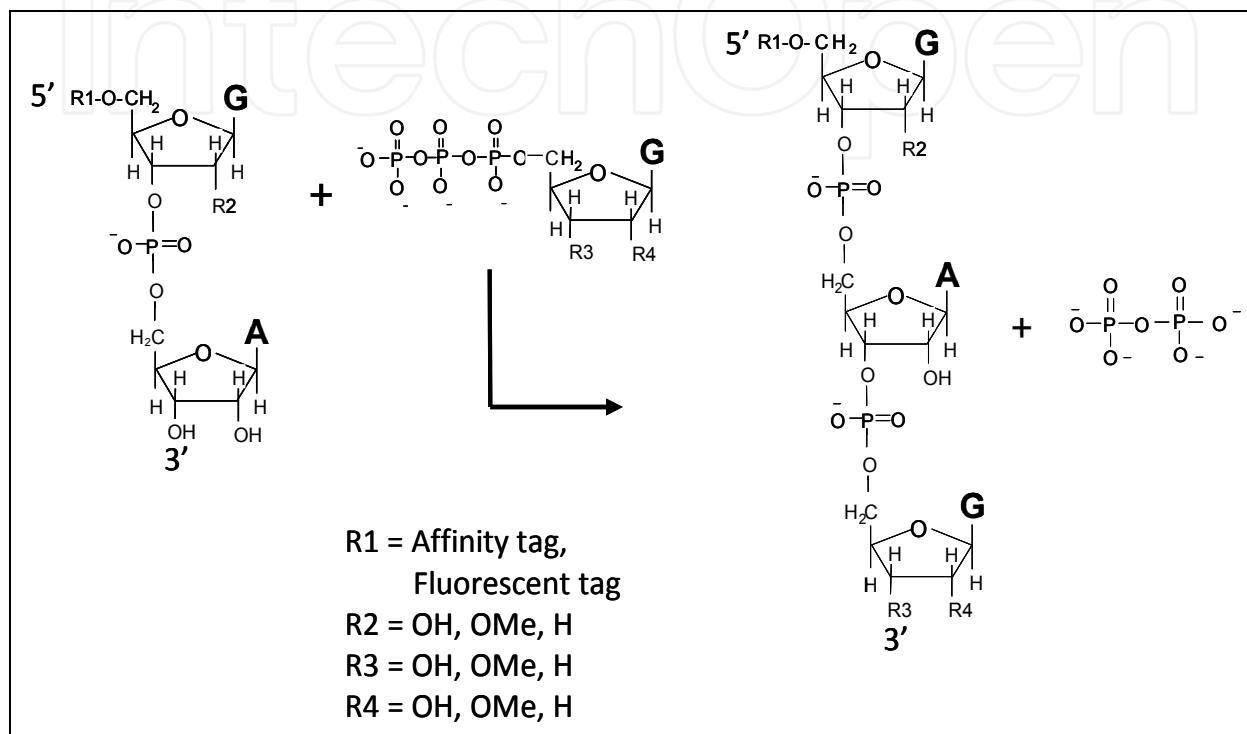

Fig. 2. Synthesis of trinucleotide abortive transcripts

Abortive transcripts can be limited to a uniform population of trinucleotides. In this example, a trinucleotide abscript is made exclusively by including a dinucleotide initiator (GpA) and a single nucleoside-triphosphate (GTP). A single polymerization step will produce a trinucleotide abortive transcript (GpApG) plus pyrophosphate (pp). The short RNA is expelled from the transcription complex and another is then made. GAG is produced at close to 10,000 copies per minute. Abscriptase is tolerant of modifications at both the 5′ position of the initiator (R1) and the 3′ position of the NTP (R3), allowing production of fluorescent or affinity tagged abscripts (such as biotin), if desired.

1.4 Abscription® – Based biomarker detection

Abscription is used to detect and quantify biomarkers in a sample by attaching an Abortive Promoter Cassette to that target (Figure 3). Once attached, Abscription is initiated and thousands of trinucleotides are generated per minute from the target. The amount of abscript produced can be quantified by analysis of the reaction by Liquid Chromatography – Mass Spectrometry (LC-MS). The substrates and trinucleotide product are separated by LC and then quantified by MS (Figure 4). The amount of abscript present is determined through the peak area off the MS.
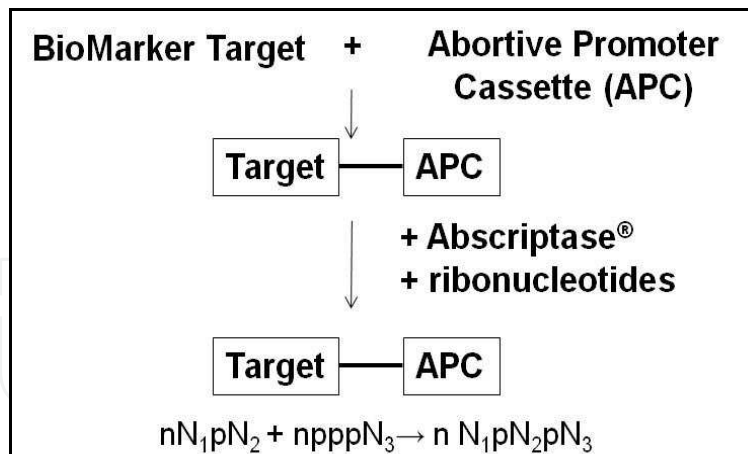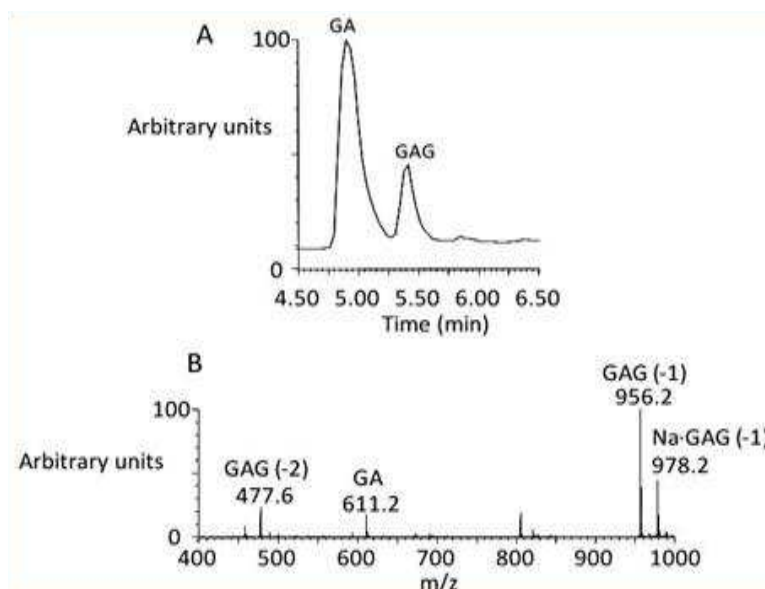
Fig. 3. Measurement of Biomarkers by Abscription®

Fig. 4. Analysis of trinucleotide abscripts by LC-MS

Abortive Promoter Cassettes (APCs) are attached to biomarkers in a sample through chemical linkage to target specific antibodies (for proteins) or through single-stranded hybridization probes extending from the APCs for direct detection of nucleic acid targets. APCs also can be incorporated into nucleic acid targets during amplification procedures through the use of APC-primers as described in Fig. 7. Abscripts are then generated from the APC attached to the target by the APC-directed, enzymatic linkage of a dinucleotide initiator (N_1pN_2) to a single ribonucleoside-triphosphate ($pppN_3$) by Abscriptase to produce a uniform population of trinucleotides and pyrophosphate. The amount of abscript is determined and used as a measure of the amount of target present.

An Abscription product is quantified as the area under the trinucleotide chromatographic peak after fractionation of an Abscription reaction on a C18 column as shown in Part A for a reaction producing GpApG from GpA and GTP. GpA and GTP are separated from the trinucleotide product GpApG. The GTP passes rapidly through the column and is not shown in the elution profile in Part A. The amount of GpApG that elutes off of the column is

determined very quantitatively by analyzing the chromatographic output by MS in terms of mass/charge (m/z). Part B shows the mass spectrum for a portion of the GpApG peak. The doubly charged, singly charged and the sodium adduct of GpApG are quantified and summed across the peak to give the total abscript yield.

1.5 Abscription – Based detection of DNA methylation: MethylMeter®

MethylMeter is a bisulfite-free assay for the quantitative detection of DNA methylation that combines affinity separation of methylated and unmethylated DNA with a target and signal amplification process called CAP (Coupled Abscription-PCR). The process is extremely rapid and works even on badly degraded DNA. Although the application is demonstrated here for the detection of imprinting orders from blood samples, MethylMeter has also been used to analyze small changes in the methylation levels in tumor DNA isolated from formalin-fixed paraffin embedded (FFPE) tissues.

1.5.1 MethylMagnet®: GST-MBD fusion protein with affinity for methylated CpG sites in DNA

MethylMagnet proteins are versatile tools for the study of CpG methylation in DNA. These fusion proteins contain the methyl CpG binding domain (MBD) of the mouse MBD2 protein fused to the glutathione-S-transferase protein (GST) from *S japonicum* (Figure 5A). The MBD from the MBD2b protein was chosen because MBD2b has the highest affinity among the known methyl CpG binding proteins for methylated CpG sites and the lowest cross reactivity with unmethylated CpGs (Fraga et al., 2003). Additionally, there are no sequence context effects on MBD2 CpG recognition, as there are for MeCP2, which requires a run of A-Ts near a CpG site, therefore a greater number of mCpG sites will be recognized (Klose et al., 2005). The linker between the GST and the MBD domains contains a thrombin cleavage site, so the MBD domain can be separated from the GST, if desired, (Guan & Dixon, 1001) although the fusion protein is active for binding to glutathione, GST antibodies, and specifically to methylated DNA (Figure 5B). The GST group contains surface cysteines that can be chemically modified to add reporters or affinity tags. Both the fluorescein and biotin labeled fusion proteins also retain the binding properties of the unmodified protein (data not shown).

The MethylMagnet protein is attached to glutathione modified magnetic beads and used to separate methylated DNA from unmethylated DNA in a genomic sample (Figure 6A). Intact genomic DNA should first be fragmented so that the CpG island of interest is physically separated from other regions on DNA that may be methylated, although MethylMagnet recognizes regions of CpG methylation density. Restriction with Mse I cuts DNA at the sequence TTAA and therefore is not affected by CpG methylation. DNA retrieved from formalin-fixed paraffin embedded (FFPE) tissues is already degraded and needs no restriction enzyme treatment before analysis. The fragmented DNA is incubated with the MethylMagnet protein which has been attached to glutathione magnetic beads. The DNA population will generally contain a mixture of methylated and unmethylated CpG sites. Methylated CpG islands will bind to the beads via interaction of the mCpG sites and the MBD domain. After capture, methylated DNA can be eluted from the magnetic beads several ways (Figure 6B).
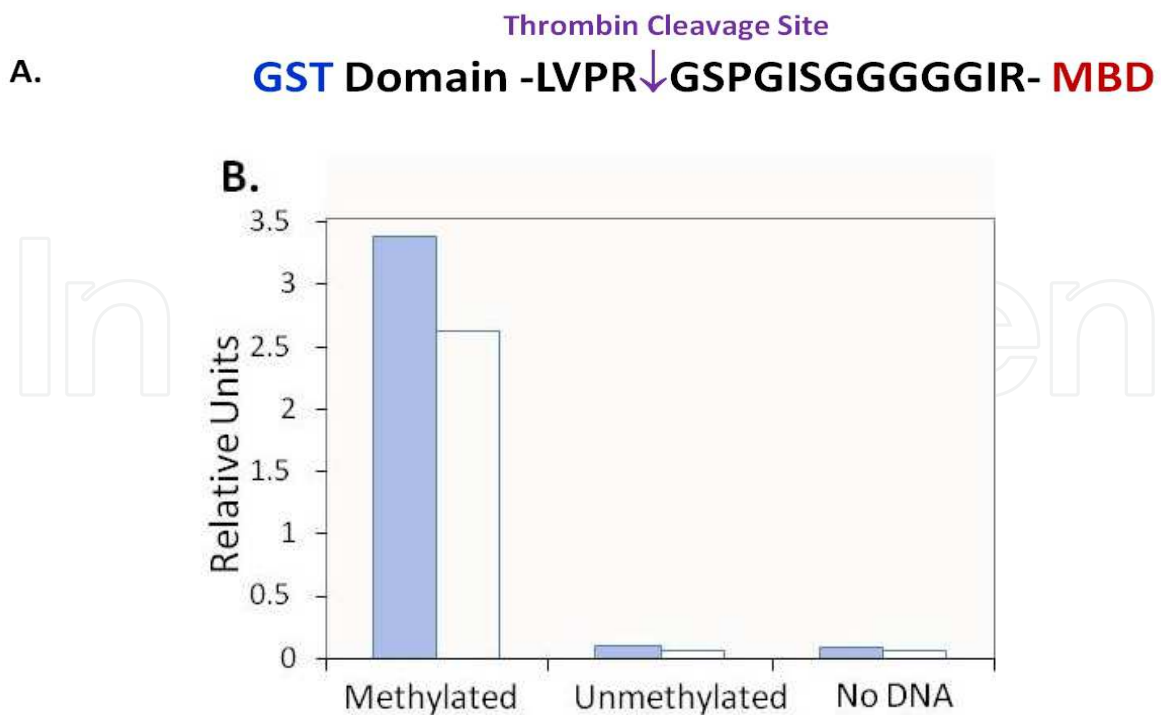
Fig. 5. Binding of the GST-MBD protein to methylated DNA A. A glutathione-S-transferase (GST) fusion protein was constructed which contains GST attached to the methyl binding domain from mouse MBD2b. The GST allows the fusion protein or its complexes with methylated DNA to be isolated on glutathione agarose or glutathione magnetic beads and eluted intact with glutathione. The GST group further allows the eluted protein-DNA complexes to be immobilized to beads or microtiter plates or visualized with GST antibodies.B. MethylMagnet proteins have much higher affinity for DNA methylated on both strands, versus hemi-methylated (data not shown) or unmethylated DNA. Purified MethylMagnet (3 nM) was incubated with 100 fmoles of a biotinylated 550 bp p16 DNA fragment that was either fully methylated or unmethylated, or with no DNA. The biotinylated DNA target was immobilized to magnetic beads. The MethylMagnet protein was incubated with the immobilized DNA. After washing, bound MethylMagnet was detected with an anti-GST-HRP conjugate. The filled and unfilled bars represent duplicate measurements. Binding was very specific for the methylated DNA fragment. The signal from unmethylated DNA was no greater than that in the minus DNA control.

1.5.2 MethylMeter™: Bisulfite-free, abscription based detection of methylated DNA

The amount of a targeted CpG island or DMR in the methylated and unmethylated DNA fractions is quantitatively detected by a new method called CAP™, for Coupled Abscription®-PCR. CAP is a combination of target amplification by PCR and linear signal generation by Abscription. Figure 7 shows the strategy for detecting DNA methylation using MethylMagnet purification followed by CAP. Methylated and unmethylated DNA molecules are separated using magnetic bead bound GST-MBD protein. Methylated DNA attaches tightly to the beads due to cooperative binding with multiple MBD domains. The unmethylated fragments remain in the supernatant fraction. The methylated DNA is eluted from the beads and then both the supernatant fraction containing unmethylated DNA, and

the eluted fraction, containing methylated DNA are analyzed by CAP (Steps 2-3). CAP involves the amplification of both the methylated and unmethylated fragments (in separate tubes) using a conventional primer and a primer that contains a single-stranded abortive promoter cassette at its 5′ end. The APC is inactive for Abscription in the single-stranded form but becomes activated when it is converted into the duplex form during the amplification of the target. The PCR reaction is supplemented with Abscriptase® along with the dinucleotide and NTP substrates for abscript synthesis.
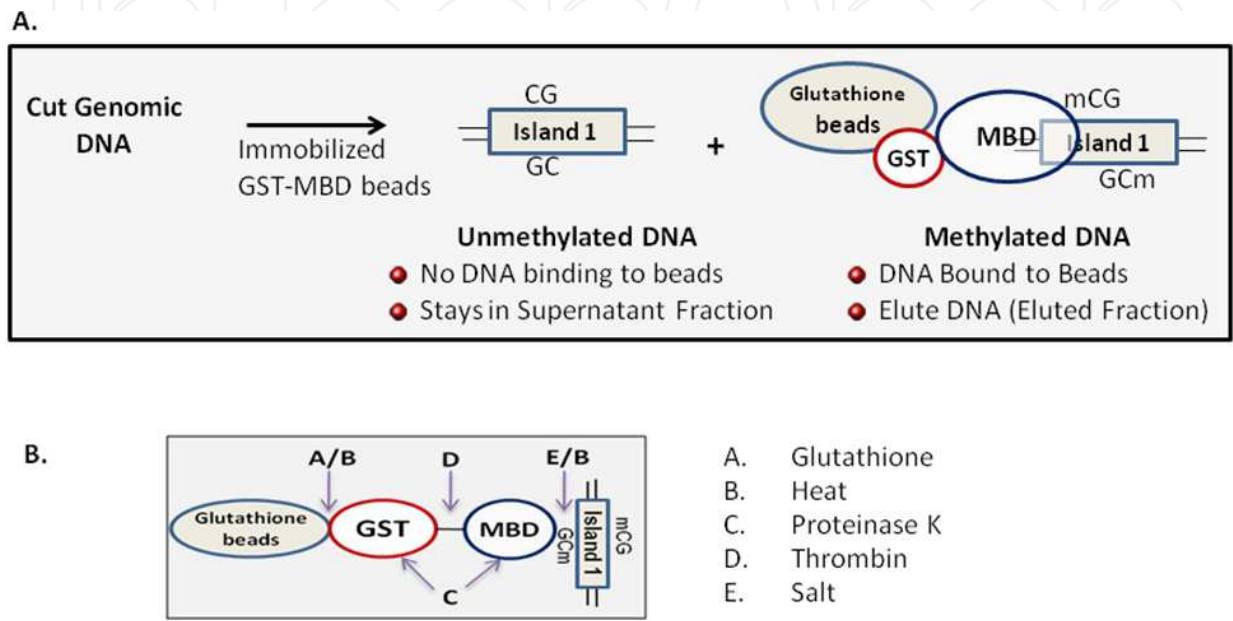

Fig. 6. Separation of Methylated and Unmethylated DNA fragments with MethylMagnet®
A. Genomic DNA is incubated with GST-MBD magnetic beads, leading to the immobilization of methylated fragments. Unmethylated DNA remains in solution and is recovered in the supernatant fraction of the binding reaction. B. The methylated DNA can be eluted from the bead several ways. Treatment with glutathione releases the GST-MBD DNA complex intact. Heat releases the DNA and denatured proteins from the beads. Proteinase K digests the proteins and releases the methylated DNA and peptide fragments. Thrombin will cleave in the linker between the GST and the MBD and will release the DNA as a complex with the MBD protein. And lastly, salt can be used to fractionate the methylated DNA based on methylation density, releasing the methylated DNA from the beads and leaving the GST-MBD protein behind.

Each new amplicon now contains the APC and begins the reiterative synthesis of trinucleotide abscripts from each target. Because Abscription generates tens to hundreds of thousands of detectable trinucleotides per each amplicon, CAP is more sensitive than PCR alone, allowing detection of DNA methylation from very little DNA without fluorescent probes and in fewer cycles than with other PCR methods. Because methylated DNA is detected based on fractionation, rather than on chemical conversion, the disadvantages of bisulfite treatment are avoided. DNA is not degraded, allowing analysis of samples as small as 100 pg to 1 ng of genomic DNA. CAP primer development is not limited by the effects of reduced sequence complexity caused by bisulfite or a requirement to overlap primers with CpG sites, as in MS-PCR.
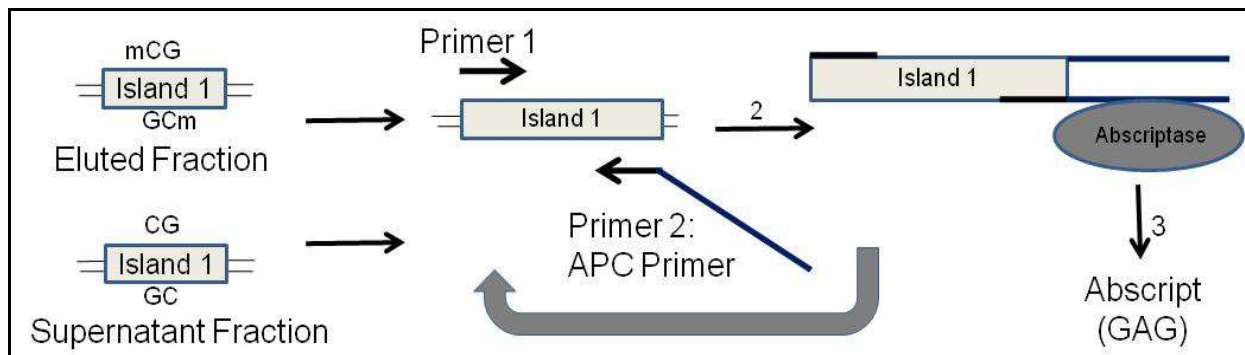
Fig. 7. Coupled Abscription®-PCR (CAP) based quantification of methylated DNA

Fragmented DNA is separated into methylated (Eluted) and unmethylated (Supernatant) fractions with the use of magnetic beads bearing the MethylMagnet® protein. The protein binds to DNA fragments with high densities of methylated CpG dinucleotides. The presence of a targeted CpG island in either the methylated DNA fraction or the unmethylated fraction is measured by coupled Abscription-PCR (CAP, steps 2-3). Targets are amplified with a conventional primer (primer 1) matched with a primer encoding an Abortive Promoter Cassette (APC) at its 5′ end (primer 2). Conversion of the APC from a single-stranded form to a double-stranded promoter activates it for Abscription. Addition of Abscriptase, a dinucleotide initiator and a single NTP allows production of the encoded trinucleotide transcript. Abortive transcripts are detected by rapid Thin Layer Chromatography (rTLC, Figure 13) or by LC-MS.

2. Results

2.1 CAP assay for detection of methylated DNA

In this study, the methylation status of a DNA segment from +234 to +539 nucleotides downstream from the start-site for SNRPN RNA variant 1 was analyzed to detect Prader-Willi and Angelman Syndrome disorders from blood. Our strategy for detecting methylated DNA involves the separation of methylated DNA fragments from the unmethylated versions through the use of the 76 amino acid mouse methyl-CpG binding domain of MBD2 fused to glutathione-S-transferase. The GST-MBD fusion protein, called MethylMagnet® was immobilized to glutathione magnetic beads to allow capture of methylated DNA fragments from a sample. After restriction, this promoter CpG island is part of a 1,237 nucleotide MseI fragment. There are 62 MseI sites separating this segment from the next upstream CpG island, two MseI sites separating it from an immediately adjacent cluster of CpG sites which probably are part of the targeted CpG island, and 179 MseI sites before the next downstream CpG island. Experiments with synthetic DNAs showed that MBD2 protein has a strong bias for densely spaced methylated sites. Synthetic DNAs with 4 methylated CpG sites spaced on average 11 nt apart were fractionated with approximately 50% efficiency. Those with six methylated CpG sites were bound quantitatively by GST-MBD2 beads while DNAs having seven to twelve methylated sites spaced between 65 to 91 nucleotides apart were bound very inefficiently (data not shown). Consequently, the linkage of widely spaced methylated CpGs that are not part of a CpG island following MseI digestion is unlikely to significantly influence the fractionation of the CpG island.

2.1.1 APC-primer development

Signal generation in the CAP assay depends on the conversion of the inactive single-stranded APC into an active duplex form. Ideally this is accomplished only when a CAP primer is copied in the course of amplifying the target. The CAP assay is potentially vulnerable to primer dimer effects that would activate the APC in the absence of target DNA. Primer-primer interactions that lead to DNA synthesis copying the APC-primer from the downstream priming segment into the double-stranded and active APC cause strong background signals independent of target DNA. The SNRPN primers were tested for this possibility by performing PCR reactions with, or without DNA over a range of annealing temperatures.

Primer	Assay	Primer Sequence (5'-3')
SNRPN F1	qPCR, CAP (APC-primer, Abscript: GAG)	TGCATAGGGATTTTAGGCGG
SNRPN R1	qPCR, CAP	CCGATCACTTCACGTACCTTC
SNRPN F2	CAP (APC-primer, Abscript: AUC)	ACCTCCGCCTAAAATCCCTATG
SNRPN R2	CAP	CTTGCTGTTGTGCCGTTCTG

Table 1. Primer sequences for CAP and TaqMan® assays

Figure 8 shows the results for one of the two well-designed SNRPN promoter primer pairs that were used to analyze patient samples (Table 1). PCR reactions were carried out for 30 cycles (2 cycles more than the standard protocol) followed by 30 min of Abscription. No background signal was seen over annealing temperatures from 62.9°C through 68.5°C (Figure 8A). Relatively strong abscript signals were generated in the presence of 1,000 copies of HeLa DNA at annealing temperatures 62.9°C and 64.9°C. Signal intensity fell at higher stringencies up to 68.5°C (Figure 8B).

Primer validation included an assessment of amplification specificity in the presence of genomic DNA. Figure 8C shows an example for the SNRPN primer pairs that were used to analyze patient samples. PCR reactions were carried out with 3,000 copies of DNA from a normal patient sample and for 32 cycles in order to allow potential non-specific amplicons to be detected by agarose gel electrophoresis. Neither primer pair produced detectable non-specific amplicons.

2.2 Analysis of the SNRPN imprinting center in patient DNAs

The MethylMeter® SNRPN assay was applied to the analysis of genomic DNA samples from 38 patients whose diagnosis of PWS and AS were confirmed based on MS-PCR of the SNRPN imprinting center (Kubota et al., 1997; Kosaki et al., 1997). A collection of 16 normal samples was also analyzed. Purified DNA samples were exhaustively cleaved with MseI in overnight digestions to unlink the targeted SNRPN promoter region from neighboring CpG islands. Treated DNAs were fractionated with GST-MBD magnetic beads without prior purification to remove the restriction enzyme. MseI was inactivated by incubating the samples at 65°C for 20 min. The supernatants of the binding reactions containing unmethylated fragments were analyzed with the paired fractions containing methylated fragments that were eluted from the beads. The eluted fractions were in the same volume and in the same binding buffer as the supernatant fractions to eliminate potential PCR
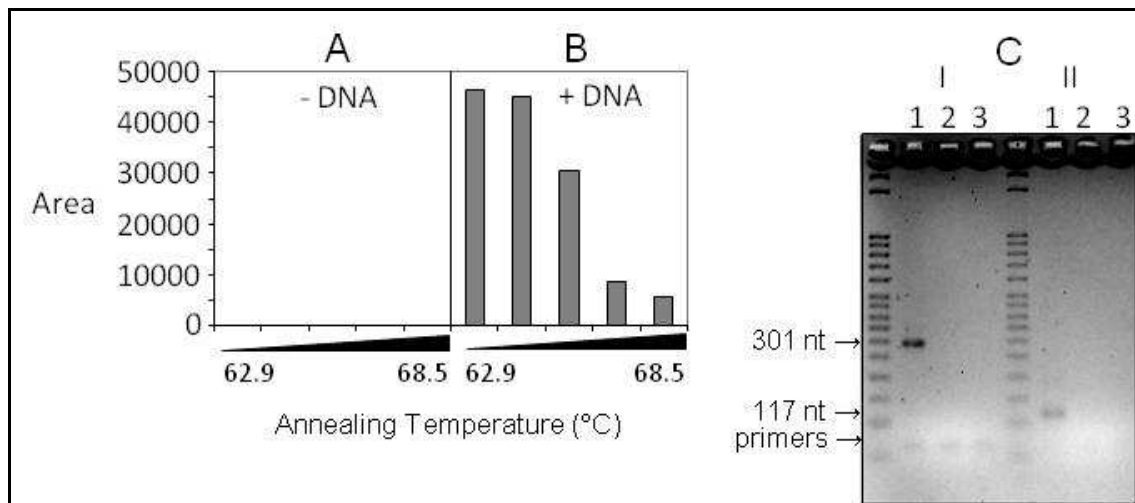
Fig. 8. APC-primer pair specificity A. APC-SNRPN F2 and SNRPN R2 were tested for specific amplification of the SNRPN and their ability to synthesize abscripts by Coupled Abscription PCR at a series of increasing stringencies in the absence and presence of HeLa DNA. Samples were analyzed by LC-MS and the total amount of trinucleotide abscript made was measured and plotted as the relative peak area. Abscription signals in the absence of DNA indicate primer dimers or self priming events that activate the APC independently of a DNA target. B. Assays in the presence of DNA indicate the optimum annealing temperature that avoids self priming without unnecessarily sacrificing abortive transcript yield in the presence of DNA. C. The SNRPN APC-primer pairs APC-SNRPN F1-SNRPN R1 (I) and APC-SNRPN F2-SNRPN-R2 (II) were used to amplify 3,000 copies of HeLa DNA for 32 cycles. Samples were fractionated in a 2% w/v agarose gel followed by staining with ethidium bromide. Lane 1 shows the amplification reaction containing DNA. Lane 2 represents the no-template control. Lane 3 shows a no-amplification control containing DNA but lacking Taq DNA polymerase. The expected amplicons contain 301 nucleotides (APC-SNRPN F1-R1) and 117 nucleotides (APC-SNRPN F2-R2)

biases due to sample buffer differences. The fractions were subjected to 28 cycles with 2 alternative sets of CAP primers with APCs that encoded either GAG or GUG. PCR reactions were followed by Abscription reactions for 15 to 30 min. The production of alternative abortive transcripts did not affect the results. The methylation status of a sample was determined by comparing abscript yields for the supernatant fraction (containing exclusively unmethylated SNRPN targets) and the eluted fraction (containing exclusively methylated SNRPN targets).

Figure 9 shows results based on abscript detection by rapid Thin Layer Chromatography (rTLC) and Liquid Chromatography- Mass Spectrometry (LC-MS). Rapid TLC had the advantage of allowing rapid processing of multiple samples in parallel. Although the results are qualitative, the imprinting disorders lend themselves to a qualitative yes-no analysis since the samples are expected to contain either all methylated, all unmethylated or a 1:1 ratio of both types of DNA. Figure 9A shows typical results for PWS (abscript GAG only in the methylated eluted fraction), AS (GAG only in the unmethylated supernatant fraction) and Normal (equal amounts of abscript GAG in both fractions). In all cases visual inspection of rTLC results for the 54 samples led to classifications of the samples in agreement with the more quantitative mass spectrometry assay (Figure 9B).
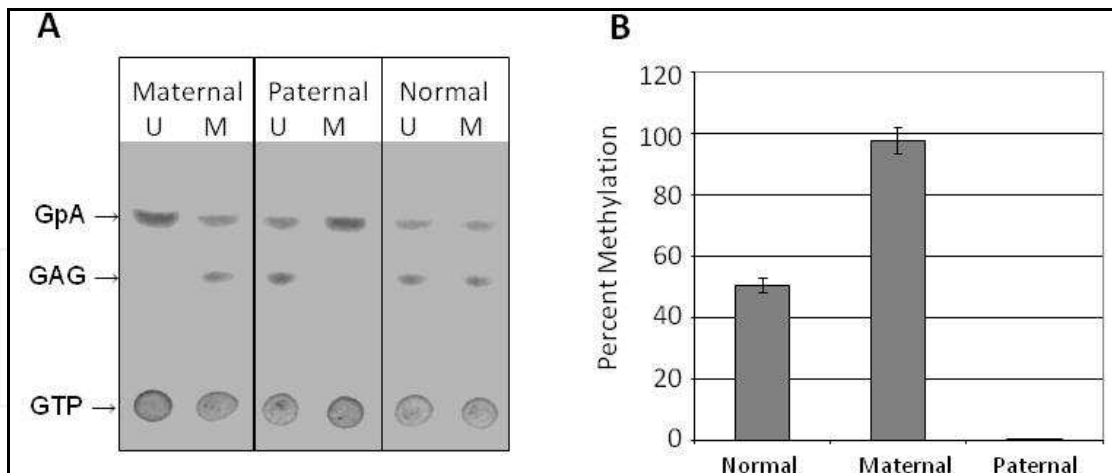
Fig. 9. CAP analysis of PWS and AS patient DNA

Patient DNAs were separated with MethylMagnet into unmethylated (U) and methylated (M) fractions. Both fractions were amplified for the SNRPN imprinting center with CAP promoter primers and then were subjected to Abscription to produce the abscript GAG. Part A shows representative rTLC results for samples showing exclusively methylated SNRPN DNA (PWS), exclusively unmethylated SNRPN (AS) or an equal representation of both methylated and unmethylated DNA (Normal). GTP remains at the origin during the development of the chromatogram while GpA and GAG are separated from each other based on their differing rates of migration in the rTLC solvent. Part B shows a quantitative summary of all the CAP assays with LC-MS detection of the abscript signals. Normal samples (n = 16) showed 50.4% ± 2.5 methylation, PWS (Maternal pattern) samples (n = 25) showed 97.5% ± 4.2 methylation and AS samples (n = 13) showed 0.23% ± 0.31 methylation. MethylMagnet inputs were between 50 ng to 150 ng of DNA.

Quantitative detection of methylated DNA was performed by LC-MS of diluted Abscription reactions. CAP reactions were fractionated by HPLC to separate the dinucleotide initiator from the trinucleotide abscript. The outflow from the HPLC column was injected into an electrospray ionization mass spectrometer (Waters LCT-Premier) to generate a chromatographic profile based on the mass/charge ratio of the abscript. The area of the abscript chromatographic peak is linearly related to the amount of abscript. Figure 9B and Table 2 show summary data for all the samples grouped into Normal, Maternal (methylated) and Paternal (unmethylated) patterns. The percent methylation was based on the abscript amount in the eluted fraction divided by the total abscript amount (the sum of the supernatant and eluted fractions). The results were highly reproducible in spite of the fact that the averages were based on multiple individuals. The large variance for the methylation level of the Paternal pattern was probably due to the statistics of sampling because the level of methylation indicates that the signals were probably generated from fewer than 5 molecules per CAP reaction based on estimates of the input amounts in calibrated CAP assays.

The analysis of patient samples also indicated that nonspecific amplification was insignificant. A non-specific amplicon that is not linked to the SNRPN target would skew the results of the normal DNA samples away from a 1:1 ratio of methylated to unmethylated target DNA. If a hypothetical secondary target was methylated the paternal methylation pattern would show a proportionately high background of methylated signal. The opposite

bias would be seen in the maternal methylation pattern if the secondary target was unmethylated. As shown below, all of the patient samples fit one of the PWS, AS, or Normal methylation patterns without significant backgrounds (Table 2). The primer sequences that were the basis for the APC-primer pair SNRPN F2, R2 assay showed no apparent bias for unmethylated DNA over methylated DNA. For example the average amplification efficiencies over a 10°C annealing temperature range were 92.7% ± 7.0 (n = 11) for HeLa DNA and 92.9% ± 5.5 (n=4) for artificially methylated HeLa in TaqMan® assays.

Methylation Pattern	Percent Methylation ± SD	No. samples	95% confidence interval
Normal	50.4 ± 2.5	16	1.23
Maternal	97.5 ± 4.2	25	1.65
Paternal	0.23 ± 0.31	13	0.17

Table 2. Cumulative results for Prader-Willi/Angelman syndrome samples

The reproducibility of the MethylMagnet fractionation method was evaluated by performing between 4 to 7 independent fractionations on DNA from 4 normal samples (Table 3). All of the fractionation runs gave good reproducibility with coefficients of variation (CVs) between 1.2% and 6.3%. The use of the alternative primers did not have a significant quantitative effect on the detection results. Sample #41 was analyzed in triplicate with APC-SNRPN F2, R2 that encoded AUC instead of GAG. The average methylation level was 49.8% ± 0.69 SD in agreement with the result for sample #41 in Table 3. The single-blinded assignments of individuals to the 3 groups based on TLC and LC-MS were 100% concordant with earlier classification of the samples based on MS-PCR (data not shown).

	[1]Cumulative Normal	Individual Normal Samples			
		#36	#39	#41	#53
Percent Methylation	50.4	50.5	47.0	49.0	46.3
SD	2.5	0.58	2.6	1.02	2.9
CV	4.9%	1.2%	5.6%	2.1%	6.3%
No. Fractionations	16	4	7	7	4

Table 3. Reproducibility of MethylMagnet fractionation [1]Data from Table 2

2.3 Sensitivity of CAP verses TaqMan®

Coupled Abscription-PCR involves the addition of Abscription, a linear signal amplification step, to PCR and is therefore much more sensitive than PCR alone. The relative sensitivity of the CAP assay was compared to a TaqMan PCR assay using the same SNRPN priming sites and PCR cycling conditions (Figure 10). Figure 10A shows a plot of Ct verses DNA amount for TaqMan amplification of HeLa DNA along with analogous plots for CAP. In the case of the CAP assay, the Ct-analog for the raw data is interpreted as the minimal cycle number required for detection of a particular input DNA copy number. These DNA amounts were not equivalent to limits of detection because the signals overshot the threshold for detection (an LC-MS signal of 100) by amounts ranging up to 549. The actual limits of detection at various cycles were calculated by extrapolating the lines relating LC-MS signals and DNA copy numbers to the detection threshold of 100 for each endpoint experiment. These

extrapolated DNA copy numbers were plotted with the associated end point cycles in Figure 10A (unfilled squares).
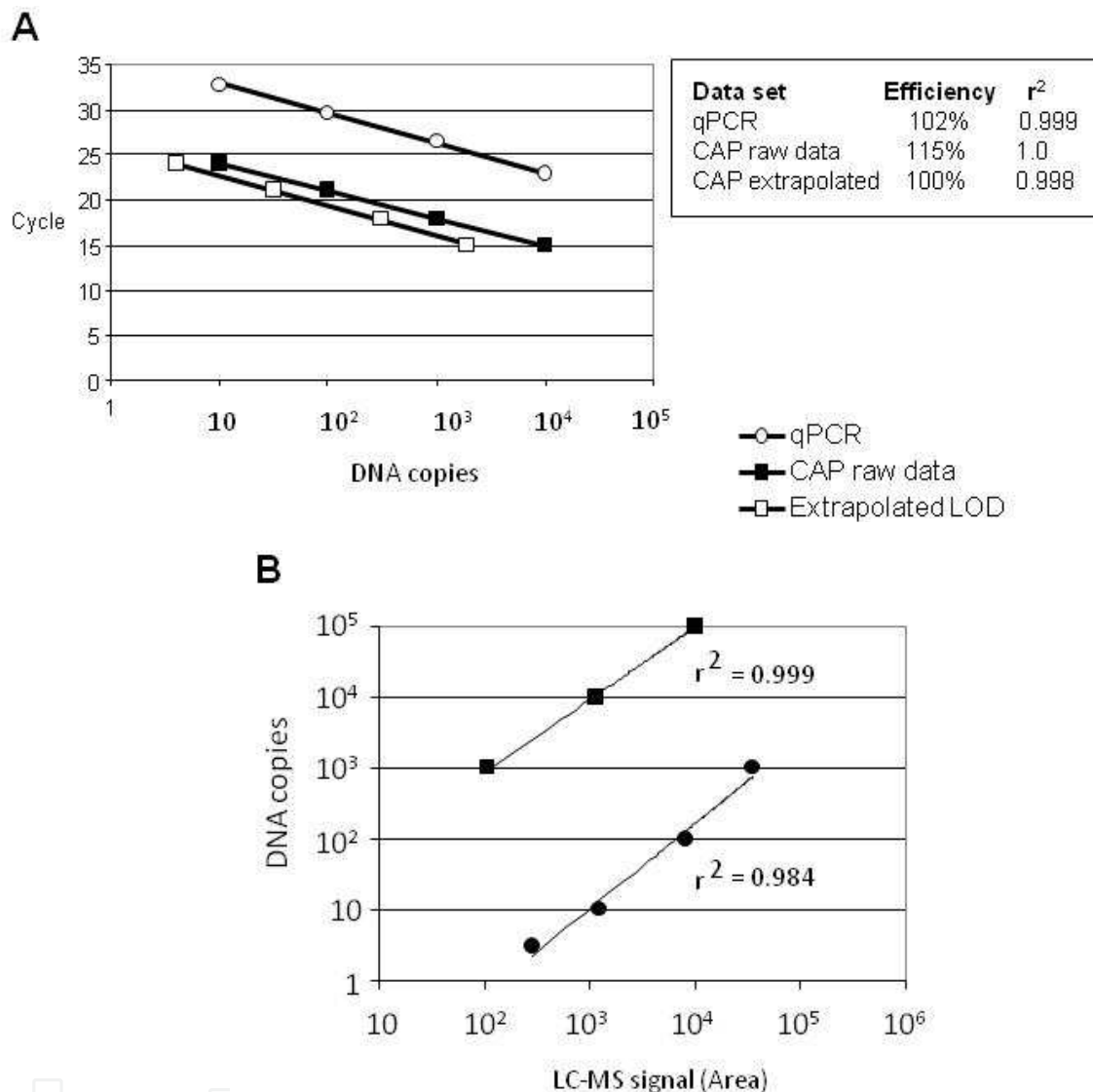

Fig. 10. Sensitivity and dynamic range of CAP. A. Calibration curves were made with dilutions of HeLa DNA. The Ct values for detection of 10, 100, 1,000 and 10,000 copies are plotted for TaqMan PCR (open circles, Efficiency: 102%; r2: 0.999). The minimal cycle number at which a plotted DNA copy number can be detected is shown for a series of end-point CAP experiments (filled rectangles, filled and open triangles). The limit of detection at a given CAP cycle number was calculated using curve fitting of the relationship between LC-MS signal and DNA copy number for each titration. The LOD was fixed to a signal intensity of 100 (unfilled squares represent the processed versions of the filled squares). Abscription reactions were for either 3 hr (squares) or for 15 min (triangles). B. HeLa DNA was titrated at the copy numbers indicated on the ordinate. Samples containing 3 to 1,000 molecules were amplified for 28 PCR cycles followed by 30 min of Abscription (circles). Samples containing 1,000 to 100,000 molecules were amplified for 20 PCR cycles followed by 15 min of Abscription (squares). Abscript signals were detected by LC-MS. Primer sequences used in the assay are listed in Table 1.

The sensitivity of the CAP assay could be adjusted with variations in the Abscription time following the PCR step. Overall a 3 hr Abscription reaction resulted in detection with a reduction of 11 cycles compared to the TaqMan assay. This translates to about a 2,000-fold improvement in sensitivity assuming 99.7% amplification efficiency. Sensitivity can also be estimated by determining the number of target copies at the detection threshold. The number of amplicons, N_t, at the Ct can be calculated by extrapolating the number of cycles to 0 in Figure 10A, where the x intercept is interpreted as the number of targets at the detection threshold (Rutledge & Cote, 2003). The qPCR assay produced an N_t of 6 x10^{11} while the normalized CAP N_t was 4 x 10^7, a 2,650-fold improvement in sensitivity with a 3 hr Abscription period. A 15 min Abscription time produced a 64-fold improvement in sensitivity over qPCR (Figure 10A).

The dynamic range of the CAP assay could be made to extend over approximately 5 orders of magnitude by performing two CAP reactions at high and low cycle numbers as shown in Figure 10B. Samples with 10,000 and 100,000 DNA copies are prone to underestimates of DNA amount at high cycle numbers due to depletion of the Abscription reagents at moderate Abscription reaction times. The high end of the dynamic range is accurately quantified by performing 20 PCR cycles coupled with 15 min of Abscription. At the low end of the range (DNA input <1,000 copies), PCR is performed for 28 cycles followed by an Abscription time between 15 to 30 min. Following this strategy we were able to consistently quantify DNA amounts between 3 molecules to 100,000 molecules.

2.4 Analysis of DNA methylation in other bodily fluids with MethylMeter

MethylMeter is an extremely robust, sensitive, rapid and quantitative method to analyze even small changes in DNA methylation levels of differentially methylated regions. It works well and reproducibly in on DNA isolated from saliva and urine (Figures A and B), in which the normal methylation pattern is 50% methylated and 50% unmethylated. In addition, the process works well and reproducibly on DNA from FFPE tissue (Figure 11C), where bisulfite based methods often fail.
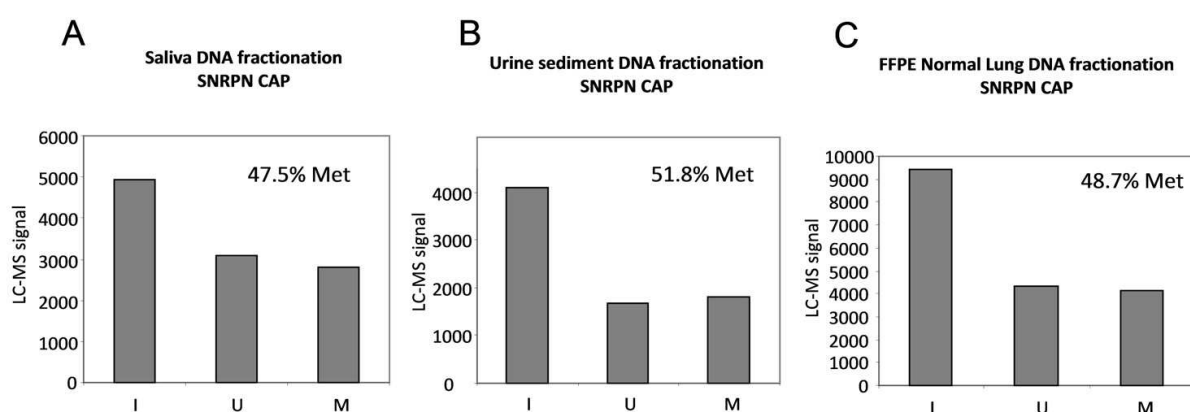

Fig. 11. Detection of methylated DNA from saliva, urine, and FFPE tumor slides. A and B. Purified normal DNAs from saliva and urine sediments (50 ng) from the same individual were separated into methylated (M) and unmethylated (U) fractions with MethylMagnet. Methylated SNRPN CpG island was detected after 28 cycles of PCR and 15 min Abscription. C. FFPE DNA from normal lung was purified from 2 glass slides (10 µM thickness). SNRPN CpG island DNA was detected after 29 cycles of amplification and 30 min of Abscription.

2.5 MethylMeter: Comparison of results to bisulfite sequencing

Although the results obtained by MethylMeter were 100% concordant with the results from MS-PCR, the method was further validated by analyzing SNRPN methylation in HeLa DNA (50%) with both MethylMeter and bisulfite sequencing. The region of SNRPN CpG island that was analyzed with bisulfite sequencing is shown in Figure 12.
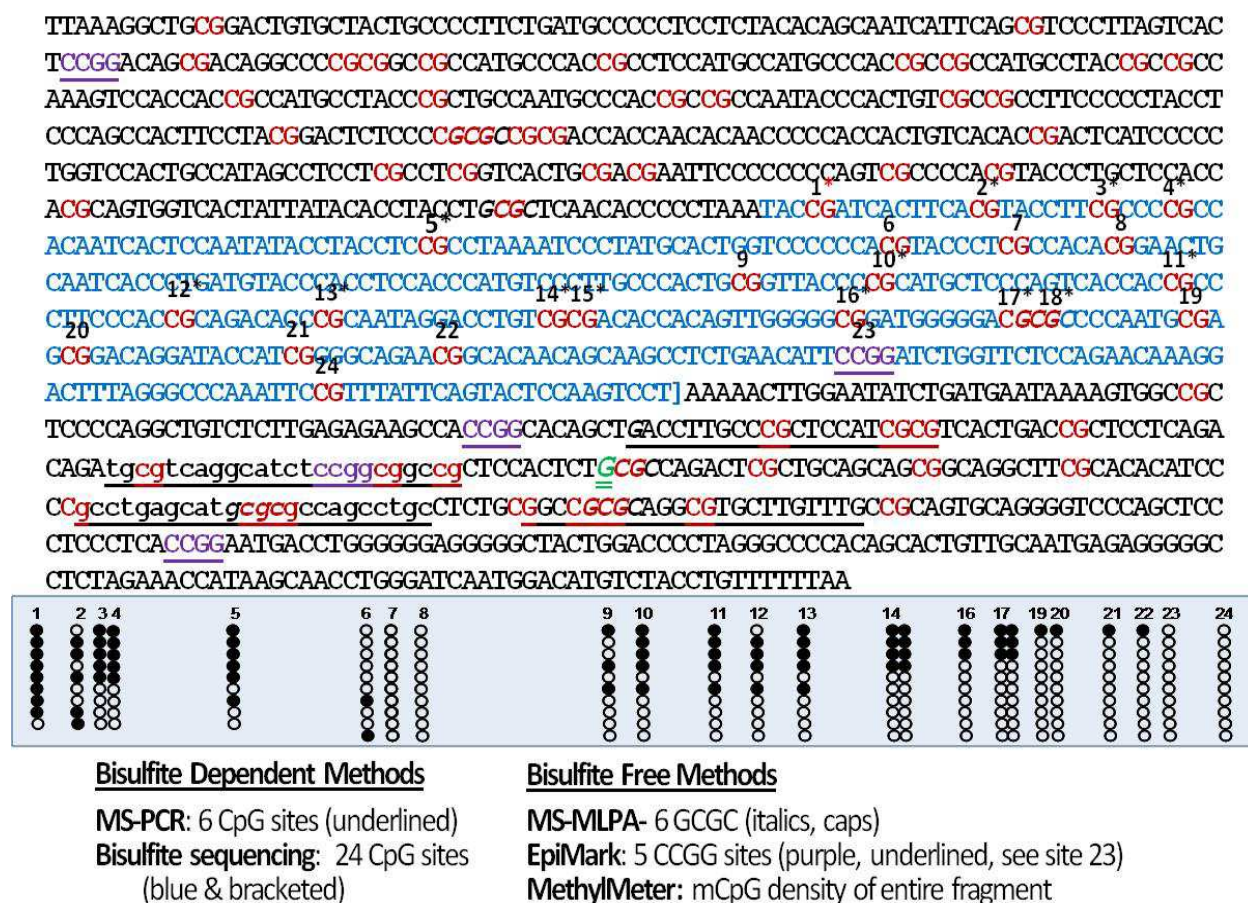

Fig. 12. Methylation of SNRPN Island: Method Comparison. The entire island probed by MethylMagnet is shown. The region that was sequenced is in the middle of the island and the CpG sites that were interrogated are numbered 1 to 24. The results of the bisulfite sequencing are shown under the sequence. A black circle indicates a methylated site. Sites that are used in MS-PCR analysis and some restriction enzyme based methods are shown. Note that site number 24, which was determined to be unmethylated by pyrosequencing, is the site used in the commercially available method EpiMark™ (NEN), which uses a restriction enzyme that cleaves differentially at this site based on CpG methylation, so this method would have incorrectly scored all of these samples as unmethylated.

The MseI generated DNA segment from +234 to +539 nucleotides downstream from the start-site for SNRPN RNA variant 1 is shown. MseI cut HeLa DNA was assayed for methylation of this SNRPN CpG island, which is imprinted and 50% methylated in normal somatic tissues. The average percent methylated DNA determined by MethylMeter was 47.7 ± 2.9%. Unfractionated HeLa DNA was sent out for bisulfite sequencing of the SNRPN segment within the MseI fragment that was probed by MethylMeter. Filled circles are

methylated CpG sites (1-24). Five islands were scored as methylated and 5 unmethylated, identical to the MethylMeter results, which took 10X less DNA and far less time and money. Sites in this island that are probed by other common methods are also indicated.

3. Materials and methods

3.1 Patient samples

Deidentified, purified DNAs from peripheral blood samples were obtained from the Molecular Genetics Laboratory, Children's Hospital and Research Center at Oakland under IRB approval. The DNA samples were previously analyzed for the methylation status of the SNRPN imprinting center with MS-PCR (Kubota et al., 1997; Kosaki et al., 1997).

3.2 Separation of methylated and unmethylated DNA with a Methyl CpG binding domain protein

Patient DNAs were fragmented with restriction endonuclease MseI. MseI cuts segments between CpG islands at high frequencies but cuts CpG islands infrequently. Fragmented DNA (7.5-150 ng) samples were fractionated with the MethylMagnet® CpG DNA isolation kit following the instructions in the user manual (RiboMed, MM101K). DNA samples were diluted 5-fold into Binding Buffer and were incubated with 5 µl of GST-MBD magnetic beads for 1 hr at 22°C with shaking at 1,000 rpm in an Eppendorf Thermomixer. Supernatant fractions were recovered after collecting the beads with a magnet. The beads were washed 2 times in 400 µl of Wash Buffer 2 with 5 min incubations at 22°C and shaking at 1,000 rpm. A third wash without incubation was performed with 400 µl of 10 mM Tris·HCl pH 8, 1 mM EDTA. Methylated DNAs were eluted from GST-MBD magnetic beads by incubation in 80% (v/v) Binding Buffer:18% (v/v) ultrapure water:2%(v/v) NEB buffer #4 at 80°C for 10 min with shaking at 1,000 rpm in an Eppendorf Thermomixer. The supernatant and the eluted fractions of the binding reactions were saved for CAP analysis.

3.3 Coupled Abscription-PCR (CAP) reaction

PCR reactions were performed with Maxima Hot Start Taq DNA Polymerase (Fermentas) using the manufacturers reaction buffer [20 mM Tris·HCl pH 8.3 (25°C), 20 mM KCl, 50 mM $(NH_4)_2SO_4$] and 2 mM $MgCl_2$, and 5% (v/v) DMSO. The dNTPS were added to final concentrations of 0. 2 µM each. CAP primers for the SNRPN promoter region were present at 1 µM each. Taq polymerase was added to 2 units/20 µl reaction. Inclusion of DMSO in the PCR reaction is essential to avoid amplification bias against methylated DNA. PCR conditions involved an initial denaturation step at 95°C for 30 sec, followed by 28 cycles of 95° for 15 sec, 64°C for 15 sec and 72°C for 30 sec. A final elongation step was at 72°C for 5 min followed by indefinite incubation at 4°C. Abscription reactions were set up by supplementing 10 µl PCR reactions with 2 µl of an Abscription master mix consisting of 3.7 x Abscription buffer (1x buffer: 40 mM HEPES pH 7.5, 40 mM KCl, 10 $MgCl_2$), 6 mM dinucleotide initiator [(GpA, GpU or ApU, RiboMed I31, I34, I14], 6 mM NTP (either GTP or CTP) and 1 unit of thermostable Abscriptase (RiboMed MME-1). Abscription was performed in a thermocycler at 77.6°C for periods ranging from 15 min to 3 hours.

3.4 Primer sequences

All CAP promoter primers were based on 2 sets of primer sequences listed in Table 1. The SNRPN F1 and SNRPN F2 had 43 nucleotide single-stranded Abortive Promoter Cassettes (APCs) linked to their 5′ ends for the CAP assays. SNRPN F1 and SNRPN R1 were also used for qPCR experiments without the additional APC sequence.

3.5 qPCR

Conditions for TaqMan® qPCR reactions were essentially identical to the PCR portion of the CAP reactions except for the inclusion of ROX reference dye to 300 nM. Primers for the qPCR experiments did not include an APC extension. The primers SNRPN-F2 and SNRPN-R2 targeted the promoter region of SNRPN transcript variant 1. A set of parallel CAP primers were derived from the same primer sequences (Table 1). The probe (5′AGGTATATTGGAGTGATTGTGGCGGG3′) was labeled at the 5′ end with 6-carboxyfluorescene and at the 3′ end with Iowa Black®FQ (IDT). Titrations of HeLa DNA from 10 copies/PCR to 10,000 copies/PCR were amplified in 20 μl volumes in an ABI 7700 Sequence Analyzer.

3.6 Abscript analysis: HPLC-Mass Spectrometry (LC-MS)

For high performance liquid chromatography-mass spectrometry (LC-MS) detection of abscripts, 10 μl of the CAP reaction was diluted into 20 μl of HPLC water in a 384 well plate. Volumes of 10 μl were injected into the LC-MS.

3.7 Abscript analysis: Rapid Thin Layer Chromatography (rTLC)

Analysis of abscripts by rTLC is both fast and extremely affordable. All that is needed is a small TLC developing tank and a handheld 254 nm light to visualize the products. Dinucleotides and trinucleotides migrate differently due to their varying polarities. Products are visualized by shining a 254 nm light on the TLC plate, which contains a fluorescent indicator. Nucleotides quench this fluorescence, resulting in a dark spot (UV shadowing). The TLC is photographed with a digital or CCD camera. Alternatively, fluorescent abscripts can be synthesized with labeled dinucleotides and then separated by TLC and visualized with a fluorescent imager or a 336 nm hand-held light (for fluorescein). Analysis by TLC involves three simple steps (Figure 13).

For rTLC detection of abscripts in the patient samples, 1.5 μl of CAP reaction was spotted 1 cm from the bottom edge of a 10 cm tall silica gel plate containing a UV-excitable fluorophore (Whatman, cat# 4420 222). The sample spots were air dried before placing the TLC plate in an air-tight rapid TLC solvent chamber (RiboMed cat# TC6-01) containing 100 ml of freshly made rTLC solvent [6:3:1 (v/v) isopropanol :ammonium hydroxide: Activation buffer 1 (RiboMed, cat AB-1)]. The plate was submerged in solvent at a depth of approximately 5 mm, with the solvent just below the point at which the reaction sample was spotted on the plate. The plate was left in the tank for approximately 20 minutes to allow the solvent to flow upwards through the TLC plate by capillary action, causing separation of the components of the CAP reaction. When the solvent was approximately 1-2 cm from the top of the plate, it was removed from the tank. Developed plates were air dried and photographed with 254 nm UV-illumination.
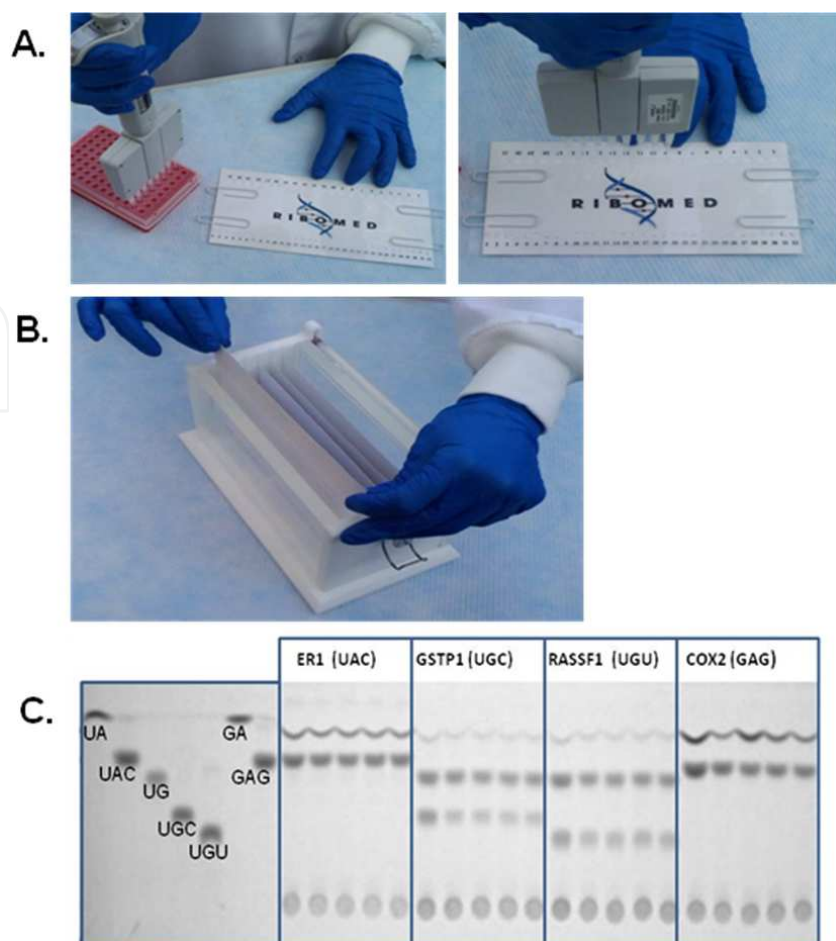
Fig. 13. Rapid Thin Layer Chromatography (rTLC) Step 1 (A). Spot samples. Samples (1 to 1.5 µl) are spotted directly onto precut TLC plates. No sample dye is required. The spotting template is designed for use with multi-channel pipettors on one side, allowing 21 samples per plate to be spotted. Even more samples can be analyzed by pipetting individually on the other side of the template, which can hold 32 samples. Step 2 (B): Put plates in the TLC developing tank. The TLC chamber holds 6 plates, so 120 samples plus 6 standards can be analyzed simultaneously using the multi-channel pipettor side. The entire process takes less than 1 hour. Up to 192 samples, or the equivalent of two 96 well plates, can be simultaneously processed when using the manual spotting template. Step 3: Irradiate the plate and to visualize the results. The TLC takes between 20 minutes to an hour to run, after which the plate is removed, air dried and visualized by UV shadowing, as shown below in (C). Spots can be quantified with the same imaging software used for gels.

4. Conclusion

Most methods for detecting methylated-CpG islands rely on chemical conversion of DNA by treatment with bisulfite. In conjunction with nucleotide sequencing this approach has the advantage that the methylation status of each CpG site in a targeted genomic region can be determined within sequence context. However, there are major disadvantages to bisulfite treatment especially when used with methods designed to determine the overall level of methylation in a genomic segment. Bisulfite conversion is carried out under relatively harsh conditions of low pH and high temperature that result in extensive degradation of the sample

DNA. This not only causes a requirement for relatively high DNA inputs, but also tends to place constraints on primer development limiting efficient amplification to small amplicons (Rutledge & Cote, 2003). Highly optimized commercial conversion kits work best with DNA samples in the range of 200 ng to 1 µg. Although samples in the range of 50 ng (15 genomic copies) can be treated, they cannot be used to generate quantitative data based on the effects of sampling statistics and losses during the post conversion clean-up (Rutledge & Cote, 2003;Ehrich el al., 2007) High cycle numbers or nested PCR are often needed to detect small amounts of bisulfite treated DNA. The reduction in sequence complexity caused by the conversion of cytosines to uracils places a further constraint on primer development by tending to homogenize the sequence compared to the untreated DNAs.

Methylation specific PCR (MSP) methods based on bisulfite treated DNA rely on sets of primers and probes that overlap CpG sites. Discrimination based on primer complementarity to CpG verses TpG is vulnerable to false positive results due to incomplete

conversion (Kristensen et al., 2008). Deep sequencing of DNA prepared by a commercial kit showed over 1% unconverted methyl-CpGs which potentially could be detected as false positives give the high sensitivity of MSP based methods pushed to high cycle numbers (Taylor et al., 2007). Heterogeneous patterns of methylation are not readily detected by MSP based methods because a consensus status must exist at all of the CpG sites encompassed by the primers and the probe. One must infer the status of an entire CpG island based on a small number of sites. A single consistently unmethylated CpG site will cause a false negative for a heavily methylated CpG island (Yegnasubramanian et al., 2006)

The use of MBD proteins to fractionate methylated from unmethylated DNA fragments avoids the complications associated with bisulfite treatment. All of the CpG sites in a CpG island can contribute to the binding of a DNA by the MBD protein. Consequently it is not necessary to infer the overall methylation status based on a few CpG sites. The MBD2 binding domain shows a strong bias in favor of DNA fragments containing multiple closely spaced methylated CpGs (Yegnasubramanian et al., 2006). This specific bias in favor of methylated CpG clusters has been exploited to survey the genome for methylated CpG islands (Serre et al., 2009).

Because MBD based fractionation does not damage DNA, less sample is required than is needed for methods that rely on bisulfite treatment. We have successfully used MBD based DNA fractionation with as little as 1 ng of genomic DNA. DNA amounts in the microgram range can be processed by scaling-up the volume of MBD-magnetic beads in the binding reaction.

The linkage of a linear signal amplification to the target amplification of PCR in the CAP method greatly increases assay sensitivity without adding complexity to assay development. Modest Abscription times between 15 min to 30 min allowed a reduction of between 6-7 cycles compared to TaqMan® assays on undamaged DNA. The CAP approach is expected to show a greater relative advantage in sensitivity compared to the application of TaqMan® assays to bisulfite treated DNA. Primer development for CAP assays is free of the constraints associated with bisulfite assays. CAP primers do not have to overlap with CpG sites nor do they have any constraints on primer spacing since no intervening probe sequence is needed. Care must be taken to avoid primer-dimer effects that lead to activation of the APC in the absence of DNA. Potential problems can be identified with conventional primer development software that shows homodimer- and heterodimer interactions. The potential for priming events can be

eliminated by changing nonconserved promoter sequences or by choosing a new reverse primer. Abscription provides an extremely sensitive assay to confirm the absence of primer-dimer effects and to identify optimal stringency conditions.

There are several options for abscript detection that depend on the nature of the targets. In the case of imprinting disorders where classification of samples is based on a simple yes or no result, a qualitative approach can be taken with rTLC. This method has the advantage that multiple samples can be processed rapidly in parallel allowing for greater through-put than LC-MS where samples are processed sequentially. All of the PWS AS samples gave unambiguous results that allowed them to be correctly classified based on visual inspection (Figure 9A). LC-MS is better suited for analysis of methylation in tumor cell DNA where quantitation is important or where it is important to monitor changes in methylation levels over time.

One potential complication of the DNA fractionation-CAP approach is associated with the fragmentation of the DNA sample to unlink the region of interest from neighboring dense clusters of methylated CpGs. Incomplete digestion is usually not a problem because numerous MseI sites are typically situated between neighboring CpG islands. However in the case where a CpG island is closely linked to a methylated repetitive element it might be necessary to use an alternative restriction enzyme or shear the DNA to very small sizes. Analysis of very small fragments should be feasible with the CAP assay because there are no assay specific restrictions on primer placement or minimal spacing between primers.

5. Acknowledgement

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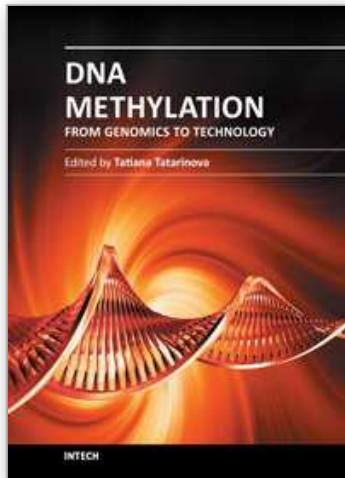
DNA Methylation - From Genomics to Technology

Edited by Dr. Tatiana Tatarinova

Epigenetics is one of the most exciting and rapidly developing areas of modern genetics with applications in many disciplines from medicine to agriculture. The most common form of epigenetic modification is DNA methylation, which plays a key role in fundamental developmental processes such as embryogenesis and also in the response of organisms to a wide range of environmental stimuli. Indeed, epigenetics is increasing regarded as one of the major mechanisms used by animals and plants to modulate their genome and its expression to adapt to a wide range of environmental factors. This book brings together a group of experts at the cutting edge of research into DNA methylation and highlights recent advances in methodology and knowledge of underlying mechanisms of this most important of genetic processes. The reader will gain an understanding of the impact, significance and recent advances within the field of epigenetics with a focus on DNA methylation.

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