Review

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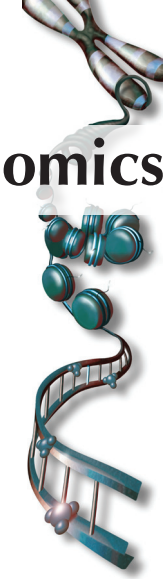
Epigenomics



Strategies for validation and testing of DNA methylation biomarkers

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DNA methylation is a stable covalent epigenetic modification of primarily CpG dinucleotides that has recently gained considerable attention for its use as a biomarker in different clinical settings, including disease diagnosis, prognosis and therapeutic response prediction. Although the advent of genome-wide DNA methylation profiling in primary disease tissue has provided a manifold resource for biomarker development, only a tiny fraction of DNA methylation-based assays have reached clinical testing. Here, we provide a critical overview of different analytical methods that are suitable for biomarker validation, including general study design considerations, which might help to streamline epigenetic marker development. Furthermore, we highlight some of the recent marker validation studies and established markers that are currently commercially available for assisting in clinical management of different cancers.

Keywords: assay validation • biomarker • bisulfite-deamination • circulating free DNA • deep sequencing • DNA methylation • MALDI • qPCR • pyrosequencing

DNA methylation testing has become a major approach in biomarker development. Several different concepts of DNA methylation testing for candidate marker confirmation and validation have been developed over the recent years. With respect to diagnostics of human disease, most efforts focus on the testing of 5-methylcytosine (5mC) within CpG dinucleotides. Although non-CpG methylation has been confirmed in stem cells in the last 5 years [1] and elucidation of biological function of modified 5mC as well as 5-hydroxymethylcytosine (5hmC) has started, the major work in the methylation biomarker field is concentrating on 5mC in the CpG context. Today genome-wide technologies are mostly used for discovery of methylation biomarkers. For confirmation of findings from the initial screenings, as well as for validation of markers in large patient cohorts, a number of different methylation testing methods are available that rely on one of the following three basic principles: bisulfite deamination, where unmethylated cytosine is converted to uracil and methylated cytosine is resistant to conversion; methylation-sensitive restriction enzymes that cut DNA depending on the presence or absence of 5mC; and affinity-based methods, using proteins for fractionation of methylated versus unmethylated DNA. Based on these three principles, we focus in this review on methods enabling quantitative analyses, which is a prerequisite for successful biomarker analyses, parallel testing of multiple markers, as well as on methods which have the capability for high-throughput analyses. Along these lines, we have compiled an overview of methods fulfilling to our best knowledge these prerequisites for optimal confirmation and validation of methylation marker panels and ideally enable the parallel testing of even hundreds of candidate markers (Table 1).

Methods & strategies for DNA methylation testing & validation

Quantitative DNA methylation analysis with methylation-sensitive restriction enzymes

One of the first technologies for the investigation of DNA methylation made use of

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Table 1. Overview and characteristics of methods for DNA methylation biomarkers testing and validation.

Method	Commercial assays (research panels) available	Bisulfite treatment	Input ng gDNA per assay (ng)	Single CpG resolution	Multiplexing	High throughput	Equipment	Dynamic range (orders)	Quantification
Bisulfite-PCR	MethyLight (Qiagen)	Yes	10–100	-	Moderate	+	qPCR	3(4?)	Calibration curve
MSRE-PCR	SA Biosciences/ Qiagen	No	10–100	-	High* (48x preamplification)	+++	qPCR	3	Calibration curve
Pyro-sequencing	PyroMark CpG assays (Qiagen)	Yes	10–100	++	No	++	Pyromark Q96ID/MD, Q24	2	Direct (%)
Deep sequencing	MethylSeq (Raindance)	Yes		+++ (and single amplicon)	High** (pooled PCR products)	+	NGS	4	Direct (read numbers)
MALDI-based DNA methylation detection	Epityper (Sequenom)	Yes	10	+	No	+++	MALDI-TOF	3	Direct (%)
MBD	Various **	No	1–100	-	Moderate	++	Various*	3	Ratio (ME/total)
	** MethylMagnet (RiboMed); MethylQuest (Millipore); MethylCap (Diagenode); MethylMiner (Life Technologies); MethylCollector (Active Motif)				* Multiplexed preamplification and single qPCR readout (not guilty for SA Biosciences kit); ** single PCRs - pooled sequencing upon barcoding MBD: Methyl-CpG-binding domain; MSRE: Methylation-sensitive restriction enzymes.		*LC-MS with Abscription, qPCR		

To achieve single CpG resolution:
- Not suited
+ Suited
++Well suited
+++ Perfectly suited

restriction enzymes (REs) [2]. Today DNA methylation analysis typically involves the use of methylation-sensitive restriction enzymes (MSREs) that cut only unmethylated DNA but not methylated DNA (e.g., *AciI, HpaII*). The combination with quantitative PCR (qPCR)-based detection enables a reliable and simple detection of DNA methylation targeting native DNA sequences. Consequently, only methylated DNA is specifically amplified during PCR [3]. In contrast to methylation-sensitive restriction enzymes (MSREs), a very limited number of REs are available that cut only methylated DNA, such as *GlaI, McrBC* and *SgeI* [4,5].

Complete digestion of the DNA is an essential step prior to amplification by PCR as even smallest amounts of uncleaved DNA are detectable and lead to false-positive results. For that reason, an appropriate assay design is mandatory. To ensure complete cleavage of the DNA, a minimal number of at least two to three cut sites is recommended within the target sequence. The combination of different MSREs is also recommended as it increases the number of possible restriction sites. Such multidigests further compensate for incomplete digestion, which might be caused by the use of just one MSRE. Additionally a combination of enrichment methods (e.g., by methyl-CpG-binding domain [MBD] proteins) and MSRE-based methylation detection may increase the sensitivity of qPCR results [6].

Many open source tools for primer design are available online (e.g., Primer3 [7]). However, the design for MSRE (q)PCR assays is more tricky, as there is no design tool available that considers the cut sites *per se*. Therefore, it is necessary to check for the number of cut sites present in a defined PCR amplicon, preferentially using a genome browser (e.g., UCSC genome browser [8]).

To qualify assays and to exclude experimental bias in every analyzed dataset, primers should be tested prior to use as suggested in MIQE guidelines by Bustin *et al.* A serial dilution covering ideally three log units per analyzed primer pair is usually sufficient to create a five-point standard curve for every primer as suggested by the MIQE guidelines [9]. The PCR efficiency, slope, intercepting point with y axis and the correlation coefficient may be given for every PCR-assay to allow an estimation of the influence of the assay performance on the qPCR data as already implemented into an updated delta Ct method by Pfaffl [10] resulting in a PCR efficiency corrected delta Ct method. In terms of MSRE-specific controls, PCR values may be corrected for input DNA amounts by subtraction from methylation-specific controls, which may be imprinted loci or even more straightforward genomic DNA fragments without MSRE restriction sites [11,12]. Comparable to qMSP every sample may also be divided into a mock and an MSRE reaction followed by qPCR, which also allows an assessment of the portion of methylated DNA for a specific locus as shown by Pulverer *et al.* [13].

Assays for methylation analysis often target sequences of high GC content, therefore optimization of the assay with different PCR enhancers like DMSO (reduction of secondary structure, facilitates amplification of GC-rich templates) or TMAC (prevents unspecific priming) is often advisable to increase assay performance. It is also highly recommended to control the DNA for complete cleavage after the digestion step by assays targeting genes with known methylation status [12].

Depending on the number of investigated targets, the digestion reaction should start with 100 ng of genomic DNA, an amount which can be easily upscaled. About 5–10 ng of digested DNA should be applied to the final qPCR reaction. It has been demonstrated that 1% of methylated DNA can be distinguished from a complete lack of methylation with an LOD of 19.58 pg methylated DNA [13]. Serial dilutions of methylated DNA in unmethylated DNA yielded recovery rates between 99 and 155%. The higher the content of methylated DNA the more precise was the recovery rate. Thus, MSRE-coupled qPCR assays are suitable for the determination of methylated DNA in heterogeneous samples containing both methylated and unmethylated fractions [11,13]. Pre-amplification protocols enable multiplexed analyses even when testing rare fractions of methylated tumor DNA in cell-free DNA is aimed.

Overall MSRE qPCR assays are an alternative to the DNA degrading bisulfite-based methods and allow accurate methylation testing.

Methyl-CpG-binding domain-affinity capture-based quantification

MBD fusion proteins bind specifically to dsDNA that is methylated at CpG sites on both strands. They demonstrate a bias for high CpG densities and preferentially extract methylated CpG islands, which are of particular interest for clinical assays (Figure 1) [14–17]. As DNA methylation is likely to be heterogeneous within an affected CpG island, validation of an affinity-based assay should target the CpG island region that was originally identified in biomarker discovery [18]. The specificity of an affinity assay is affected by fragmentation of the DNA prior to fractionation, which unlinks the region of interest from neighboring CpG sites whose methylation status could bias the fractionation. Isolation of intact islands can usually be achieved with restriction endonucleases that recognize sites that contain only A and T.

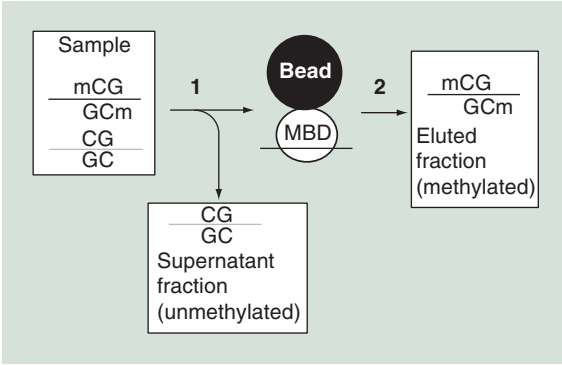

Figure 1. Methylated DNA fractionation with immobilized methyl-CpG-binding domain proteins.
A sample containing a mixture of methylated and unmethylated fragments is incubated with an MBD-domain protein that is immobilized to magnetic beads. Methylated fragments bind to the bead via the MBD protein (step 1) and are released in an eluted fraction by denaturing the protein or by increasing the NaCl concentration (step 2). The supernatant of the binding reaction contains the unmethylated fragments.
MBD: Methyl-CpG-binding domain.

MBD beads are expected to have different binding capacities for different fragments in a genomic DNA preparation because fragments with different CpG contents will compete for binding with different efficiencies, which becomes important at very high DNA inputs. A standard MBD bead volume should be defined by titrating MBD beads at the maximum DNA input using a sample that is methylated in the target region. The stringency of the binding reaction is an important variable, particularly if the target has few CpGs. Stringency is controlled by NaCl concentration in the binding and wash buffers.

After MBD separation of the methylated and unmethylated target fragments, the extent of methylation of that target is determined by quantifying how much target is present in the eluted versus bound fractions. PCR bias against methylated DNA can affect PCR efficiency, especially in the early cycles of amplification [19], so Tm-lowering additives should be included at concentrations that minimize the difference in PCR efficiency between unmethylated and artificially methylated samples [6]. Optimizations of denaturation conditions (temperature and duration) can be applied to first three cycles, when methylated strands comprise a significant fraction of the template pool, after which cycles can be performed under conditions that have been recommended for the amplification of unmethylated G+C-rich DNA [20].

Recommendations on targeting and PCR optimization apply equally to all commercially available MBD-affinity kits. Kits differ in other ways based on the design of the fusion proteins. Those based on glutathione-S-transferase (GST) are dimeric (MethylMagnet, RiboMed; MethylQuest, Millipore; MethylCap, Diagenode), while those with hexa-his affinity tags are monomeric (e.g., MethylMiner, Life Technologies; MethylCollector, Active Motif). Stringency and binding capacity optimizations for one commercial kit are not necessarily transferable to another.

Assay controls confirm the effectiveness of the binding and elution steps. A genomic DNA that is unmethylated for the target and an artificially methylated positive control can be processed in parallel with the test samples. An alternative control for the fractionation step is to assay for an imprinted gene in a normal sample, which should be 50% methylated.

MBD affinity assays have a large dynamic range. Successful fractionation is possible with less than 0.1 ng of genomic DNA, after eliminating nonspecific binding to the bead matrix [17]. The upper limit is controlled by the bead input volume.

Sensitivity is a major issue for the analysis of formalin fixed paraffin embedded (FFPE) DNA samples because the majority of the DNA is unamplifiable due to frequent crosslinks and strand breaks. Bisulfite-based methods tend to aggravate this problem because the bisulfite treatment adds further damage to the DNA sample. Affinity-based methods avoid this complication because the DNA is simply purified and fractionated. Amplifiable DNA in FFPE samples can be successfully fractionated in the presence of large amounts of damaged DNA [21]. The following recommendations for processing FFPE samples are based on our unpublished experiments:

- Some commercial kits for FFPE DNA purification suggest incubation temperatures as high as 90°C to reverse crosslinks and inactivate proteinase K. These conditions will likely denature the DNA making it unsuitable for MBD-based affinity fractionation. Incubations for crosslink removal or enzyme inactivation should not exceed 80°C;
- Even though FFPE DNA contains a high frequency of single-strand breaks, duplex DNA segments are long enough that targeted CpG island regions must be unlinked from neighboring CpG sites with restriction endonucleases to avoid possible bias in the fractionation step. While crosslinks can be removed by heat treatment, strand breaks must be compensated for by limiting amplicon sizes to less than 200 nt;
- The total DNA input amount should be taken into account in order to determine the minimal MBD-bead input to avoid competition for binding. The amplifiable DNA fraction can be below 1% of the total DNA content;

- Our experience with the MethylMagnet kit is that methylated DNAs from FFPE samples are not efficiently eluted from MBD beads by simple heat denaturation of the MBD protein. Reliable measurements can be made by direct amplification from the beads.

The sensitivity of the assay is influenced by the downstream detection method. Quantitative PCR is satisfactory for undamaged DNAs but might require excessive numbers of cycles or nested PCR to analyze highly damaged DNAs. Amplification via CAP (coupled Abscription-PCR) uses promoter-linked PCR primers followed by Abscription (Abortive Transcription) and is up to 1000-fold more sensitive than qPCR [21]. Adding signal amplification to target amplification makes clinically important FFPE samples more accessible.

Bisulfite-based quantitative PCR testing

Bisulfite treatment of genomic DNA provides a suitable option to distinguish methylated from unmethylated cytosine residues by different downstream applications [22]. Several quantitative assays have been developed in order to interrogate the methylation status of selected loci. Generally, two different assay setups can be distinguished: either PCR reactions are performed to amplify bisulfite-converted DNA irrespective of methylation status (MIP) (no CpGs in primer sequence) or PCR reactions are methylation specific (MSP) (CpGs within primer sequence).

The first established quantitative MSP assay was MethyLight, which employs MSP combined with methylation-sensitive probing (TaqMan®, Life Technologies, Carlsbad, CA, USA) [23]. This highly sensitive assay is capable of detecting methylated DNA in a 10,000-fold access of unmethylated DNA, which makes it suitable for the analysis of complex heterogeneous material such as clinical samples. Several quality control reactions controlling for sample integrity and quantity, sample recovery after bisulfite conversion as well as bisulfite conversion efficiency are advisable for assay setup [24]. In order to specifically amplify methylated DNA primers and probe should contain one to five CpGs, which can be designed using MethMarker, a platform for the design and optimization of gene-specific DNA methylation assays [25]. Four PCR reactions using two different samples are needed to determine the methylation level of a selected region; the bisulfite converted DNA of the sample of interest and bisulfite converted 100% methylated DNA as a reference (*in vitro MSssI* treated) are amplified using methylation-specific primers and probe for the gene of interest plus a methylation independent, bisulfite conversion specific set of primers and probe for a reference locus such as the repetitive ALU-C4 locus to control for DNA input. The methylation level is then calculated using the PMR (percentage of methylated ratio) value, which gives a relative measure of DNA methylation, based on a standard curve of a dilution of *MSssI* treated completely methylated DNA [24]. MethyLight assays can be multiplexed using different fluorescent-labeled probes allowing for high-throughput applications [26]. A higher grade of sensitivity and accurate quantitation can be obtained by employing digital MethyLight, which allows for the detection of single-molecule DNA methylation [27].

This methodology is found in the literature frequently as quantitative MSP (qMSP) and seems to be a method of preference when testing targets in clinical specimens such as bronchial washings [28] and serum [29] and biopsy material [30].

A more economic variation of MethyLight represents sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP), which relies on probe-free MSP using DNA intercalating fluorescent dyes combined with high-resolution melting (HRM) analysis, which allows for the identification of false positives [31]. Evaluation of melting curves subsequent to PCR amplification provides information relating to the specificity of the reaction. Incomplete bisulfite conversion and false priming yields shifted melting curves compared with fully methylated samples and provides an additional quality control. Heterogeneous DNA methylation can also be detected based on altered melting curves providing an advantage compared with conventional MethyLight, which only detects highly methylated sequences. As for MethyLight, the methylation level of a gene of interest is determined based on a methylation-independent internal control and a calibrator sample of 100% methylated DNA. Dilution of the methylated standard down to 0.1% could be reproducibly detected, thus resulting in sensitivity comparable to MethyLight.

Two further assays with high analytical sensitivity include HeavyMethyl and MS-HRM, which are both based on methylation-independent priming of bisulfite-converted DNA [32,33]. HeavyMethyl uses blocking oligos, binding to unmethylated DNA, which overlaps with primer-binding sequences, thus allowing for amplification of methylated sequences only. Quantification of methylated DNA is obtained by fluorescent probing and by using PMR calculations analog to the MethyLight approach. HeavyMethyl assays were reported to reach a relative sensitivity of 1:8000, detecting a few copies of methylated DNA in 400 ng of nonmethylated background DNA [32]. The HeavyMethyl assay is used for the commercially available kits 'Epi proColon' and 'Epi proLung' by Epigenomics AG to detect DNA methylation in colon and lung cancer, respectively [34,35].

Methylation-specific high-resolution melting (MS-HRM) analysis, which also uses MIP to amplify bisulfite-converted DNA, quantifies methylation levels based on melting profiles of amplicons of bisulfite-converted DNA. Highly methylated sequences contain a larger number of CpGs in their sequence and therefore have higher melting temperatures compared with less methylated samples, which contain more TpGs and thus shifted melting profiles. Methylation levels are estimated based on melting curves of PCR products of standards with known methylation status and methylation levels as low as 0.1% can be detected [33]. Drawbacks of this method might be difficulties in interpreting melting curves of heterogeneous methylated samples.

In sum, bisulfite-based qPCR is highly sensitive and suitable for high-throughput analyses and some assays are already in use for clinical testing.

Pyrosequencing-based methylation analysis

Pyrosequencing is a highly versatile methodology offering significant advantages in DNA methylation analysis [36]. The main advantages of pyrosequencing-based methylation analysis are the ability to quantitatively interrogate multiple CpGs, include multiple bisulfite controls, being a fast and medium cost method. Limitations are largely due to the low temperature (28°C) of the reaction, which enables the formation of many secondary structures; this is why careful assay design and template optimization are very important for successful assays.

A brief description of the method is given in Figure 2. Every CpG position is interrogated by a sequential injection of C and T (or G and A if reverse sequencing primer is used). The methylation percentage is determined by the relative incorporation of the two nucleotides at the site. A representative pyrogram is shown in Figure 3.

Assay design demands unique software (Pyromark Assay Design 2.0, Qiagen Venlo, Netherlands). It generally requires some experience to achieve optimal designs, as the automated primer selection function rarely results in good designs for DNA methylation, while it works well for the SNP assays, for which it was originally designed. In addition, the Tm calculation in

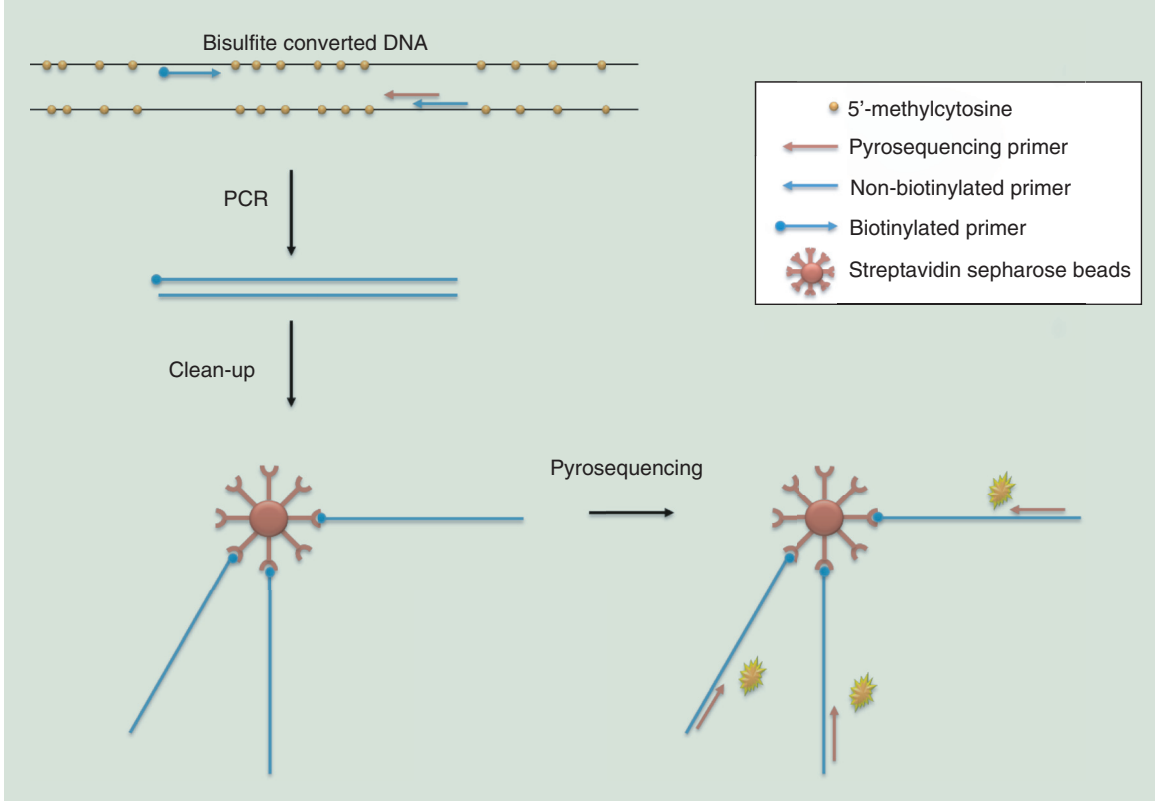

Figure 2. Schematic description of pyrosequencing-based methylation analysis work flow. The CpG-bearing sequences of interest are amplified by PCR, after bisulfite conversion, utilizing flanking CpG-free regions. One of the PCR primers is biotinylated at its 5′ end allowing for a fast (~10 min) post-PCR clean-up involving streptavidin sepharose beads. Following clean up, the single (biotinylated) strand immobilized on the beads is added in the reaction plate containing the annealing buffer and sequencing primer and the plate inserted in the equipment for the reaction to commence. The remaining reagents are dispensed in a predetermined manner by the equipment through a cartridge.

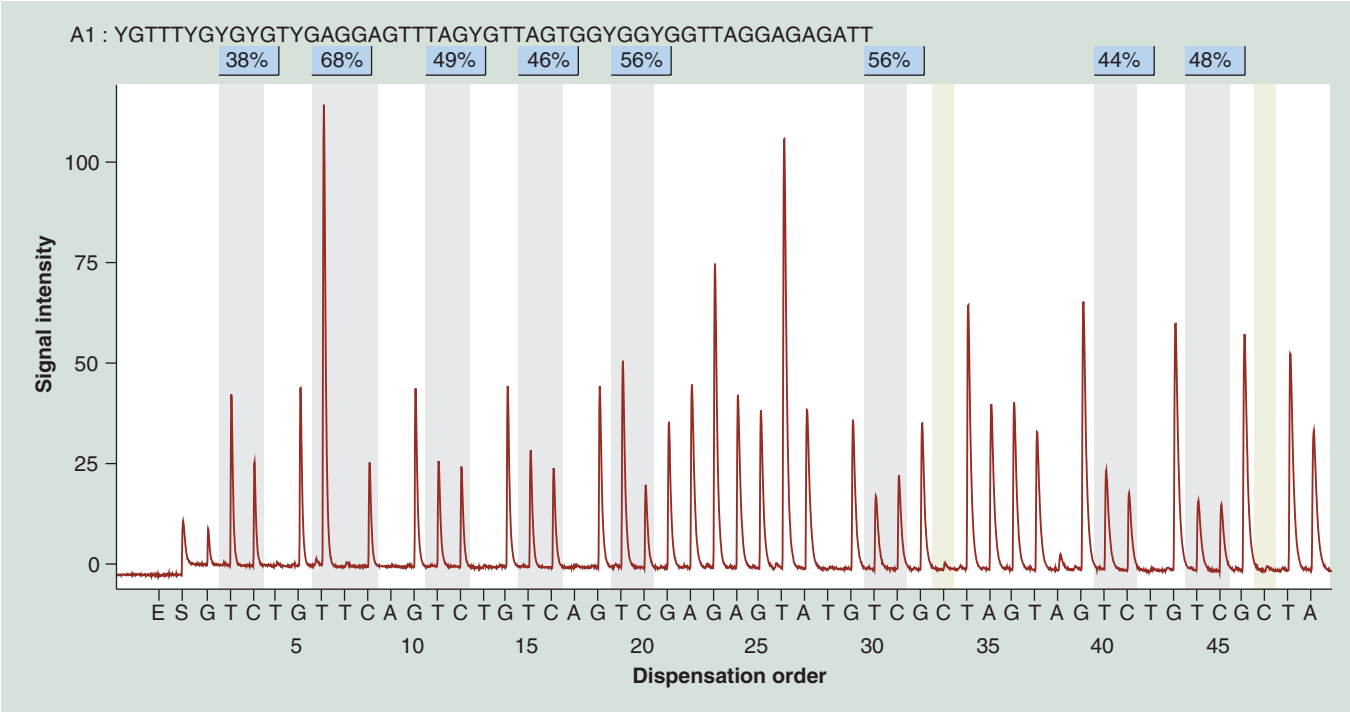

Figure 3. Example of pyrogram from pyrosequencing-based methylation analysis run. Interrogated CpGs are indicated by the gray lanes. The percentages in the boxes above demonstrate the degree of methylation detected. The color of the box reflects the quality control result (blue = pass, yellow = check, red = fail). Percent methylation (%) is calculated by the software as [C/(C+T)] for each CpG dinucleotide. The C dispensations at positions 33 and 47 (yellow lanes, corresponding to C not within a CpG dinucleotide) are for bisulfite conversion quality control purposes.
For color images please see online www.futuremedicine.com/doi/full/10.2217/EPI.14.43

this software is not optimal (to the author's experience) thus additional software (e.g., Primer Express) should be used for this purpose. Here are some brief guidelines for the design:

- The read length is normally 60–70 nt, although >100 nt is achievable in optimal designs. Thus the amplicon length should be kept fairly short; definitely below 300 bp and optimally up to 150 bp;
- The primers normally cannot include CpGs and should be avoided if possible. Successful designs may utilize primers with a mismatched nucleotide (G/A) or a mixed nucleotide (C/T, also known as 'wobble') at such a C position within the three to four 5′ positions of a 22mer for example. However, appropriate optimization and validation have to be undertaken to eliminate or minimize the potential bias for methylated or unmethylated target copies;
- Optimally, PCR primer length should be between 18 and 24, with Tm of no less than 48°C (calculation for 200 nM oligo, 50 mM K+). Tm should also not exceed 65°C, but this is extremely unlikely to be met when targeting bisulfite-converted DNA. Typically for PCR, primers should not differ more than 2°C in Tm;
- Although homopolymers should be generally avoided within PCR primers, this rule will be frequently compromised in bisulfite DNA related designs. Still, if it cannot be avoided, homopolymers should be limited at the 5′ of the primer.

Once somebody gets experience, the success rate of designed assays can be over 95%.

Successful pyrosequencing is heavily dependent on the quality and quantity of the PCR product. Too little product will result in high noise-to-signal ratio, while too much will end up in peak tailing and possible loss of its quantitative efficiency. Typically, PCR optimization for pyrosequencing involves testing dilutions of the biotinylated primer into nonbiotinylated, ranging from 1:1 to 1:2. The use of lower amounts of biotinylated primer reduces the competition of the unused excess primer to the PCR product and therefore enhances the signal. The thermal profile has of course to be optimized, using the highest possible temperature that does not compromise yield. The PCR product has to be checked by agarose electrophoresis and should be free of artifacts, primer dimers, etc. No other optimization, beyond PCR amplicon quality/quantity, is required for the pyrosequencing reaction itself.

The performance and linearity of the assay has to be shown ahead of screening by the inclusion of a stan-

dard curve of artificially methylated (normally by *SssI* methyltransferase) into unmethylated DNA. It has to be noted that DNA methylation is tissue specific and therefore normal tissue DNA cannot *de facto* be assumed to be unmethylated for every gene/sequence. Synthetic (whole genome amplified) DNA is preferable for this negative control (unmethylated) purpose.

Data analysis is one of the major advantages of pyrosequencing: it is automated, taking no more than 1 min. No data preprocessing is required.

As mentioned above, assuming assay design is sound, pyrosequencing performance depends solely on the quality and quantity of the PCR product. DNA inputs between 10 and 100 ng in the PCR reaction are expected to provide similar results in the pyrosequencing reaction. The nature of the method does not allow for multiplexing, however one can potentially run 96 different assays per plate; of course this requires 96 different PCR products. As a typical post-PCR method utilizing nonallele-specific primers, pyrosequencing can reliably detect DNA methylation down to 5%. Lower levels will be associated with very high variability.

The above-mentioned characteristics make pyrosequencing-based methylation analysis ideal for screening sequences in primary disease tissue, either in single-target approaches or, frequently, in technical and biological validation of DNA methylation microarray results. To date, it is considered as the gold standard technique for this purpose and has contributed to a very large number of studies [28,37–39].

Targeted deep-amplicon bisulfite sequencing (TDBS)

Bisulfite sequencing applies different sequencing methods on bisulfite-treated genomic DNA in order to determine the methylation status of CpG dinucleotides. Initial sequencing methods used subcloning to combine positional information with allelic information. Hereby typically 10–100 clones or alleles were sequenced. With the availability of Next Generation Sequencing technology, which provides a clonal read out, deep sequencing upon bisulfite conversion has become a very effective approach for analyzing methylation patterns. As far as to analyses of clinical samples are concerned, heterogeneity of sample material due to both biology as well as due to sample preprocessing (e.g., formalin fixation) has forced investigators to increase the sequencing depth which would have been almost impossible using the classical cloning approach but is easily achieved using today's massive parallel sequencing options [40]. The massively parallel sequencing can easily and directly sequence greater than 100 bisulfite PCR products in a single sequencing-run without subcloning. This technology also shows high robustness, and superiority with respect to multiplexing of, for example, 25 gene-related CpG-rich regions from more than 40 individual samples in a single sequencing run [41]. Today's limitation of TDBS is rather the capacity for setting up of PCR assays for multiple candidate loci, whereas analyzing different patient samples is easily achieved when a pool of amplicons from different individuals is ligated with barcoded sequencing adapters during TDBS sample preparation.

For design of assays, recommendations already given for bisulfite-specific PCR amplification in previous sections should be considered. As for pyrosequencing, the primer sequences have to be designed spanning the region of interest and avoid CpGs within the primers. Due to lower complexity of bisulfite-treated DNA, it is more difficult to find suitable primers. However for specific target amplification software like MethPrimer [42] design program, which is based on the popular Primer3 [7] program, supports both MSP and bisulfite sequencing primer (BSP) design. Another example of primer design software for bisulfite-converted DNA is BiSearch [43]. The algorithm is not based on other primer design software but starts from an own implementation of the nearest-neighbor method to calculate the melting temperature of the DNA strands. An important feature of BiSearch is the 'specificity check', in other words, the algorithm uses a simple search method to find other targets of the primers in the bisulfite-treated reference genome. MSPprimer [44] generates possible primers based on a sliding window. The specificity check of the primers is based on the specificity-determining subsequence (SDSS) theory [45]. The SDSS is the smallest 3′ subsequence of a primer for which the fraction of the template associated with that subsequence exceeds a given threshold.

For precise quantification of methylation ratios, deep amplicon sequencing requires a methylation-independent amplification of the targeted regions [46]. In practice, methylation-independent assays are often hard to achieve due to PCR bias, which favors the amplification of unmethylated sequences. Optimization with positive (methylated DNA) and negative (unmethylated DNA) control samples is therefore highly recommended. These controls are available as commercial kits or can be generated from a DNA source [47].

Sequenced bisulfite-treated DNA is mostly devoid of cytosines, which means they will not map without mis-matches to a standard reference genome. Most algorithms [48] unmethylate *in silico* the remaining cytosines before mapping to the unmethylated reference amplicon, and reconvert the methylated sites to cytosines after mapping.

Comparable to a normal MSP, a limited amount of input bisulfite DNA (10–100 ng) is required for deep amplicon sequencing. Multiple assays per sample can be done in multiplex, allowing for testing of multiple markers in multiple samples.

Quantitative DNA methylation analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Region-specific quantitative DNA methylation analysis can be performed using base-specific cleavage coupled to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [49–51]. This methodology (EpiTYPER analysis) enables high-throughput assessment of DNA methylation within target regions, enabling the quantitative assessment of methylation levels for the majority of CpG dinucleotides across a region of interest [52–54].

Proper assay design is a prerequisite for robust performance and consistent interpretation of DNA methylation levels. Two distinct parameters govern assay design: the source, quality and quantity of the DNA and factors linked to the assay itself. The quantity of amplifiable DNA present during the PCR reaction directly contributes to the quantitative accuracy of an assay [55]. The process of treating DNA with sodium bisulfite results in the degradation of DNA, reducing the amount of effective starting material proportional to the length of the downstream amplicon/assay [56]. When combined with long amplicons or coupled to chemically, biologically or otherwise fragmented input material, the impact of this treatment can ultimately result in the dramatic reduction of the number of amplifiable DNA molecules. For full-length genomic DNA, current bisulfite treatment methods should result in a sufficient number of amplifiable copies even at amplicon lengths reaching 500–600 bp; however, for fragmented DNA including circulating (cell free) cf DNA or FFPE material, unpublished data suggest that amplicon sizes should be reduced to 100–200 bp.

EpiTYPER assay design is augmented by the use of the EpiDesigner assay design tool [57]. This tool facilitates the process-including assay selection, oligonucleotide ordering and downstream assay processing. Primers are designed to hybridize to regions devoid of CpG dinucleotides but containing cytosine residues. These parameters enhance amplification of bisulfite-converted DNA strands while mitigating methylation-dependent amplification, allowing quantification of approximately 82% of CpG dinucleotides located in CpG islands using a single cleavage reaction and yielding assay success rates of greater than 90% [49].

Subsequent to bisulfite conversion, a region-specific PCR reaction is performed to enrich for the region of interest and incorporate a polymerase recognition sequence. Due to differences in assay specific parameters, optimization of PCR conditions should be performed to ensure robust yield. The amplified product is then subjected to a simultaneous *in vitro* transcription reaction and base specific cleavage using RNase A with the products measured using MALDI-TOF mass spectrometry (Figure 4). This downstream process contributes a minority of the assay variance [55], enabling consistent downstream reaction conditions amenable to automation.

As described previously, the quality of the methylation assay is dependent upon both the assay and the input material. For assay-specific controls, template consisting of *in vitro* unmethylated, methylated and a mixture of the aforementioned sample types can be used to quantitatively assess the methylation values for the assay [49]. For sample qualification, EpiTYPER also provides a method enabling the rapid assessment of the quality of the input material [55]. Ensuring the quality of both the assay and input material enhances the likelihood of robust, reproducible assay performance.

The interpretation of the data from the MALDI-TOF mass spectrometer is enriched through the use of the EpiTYPER software. This software computes the methylation ratio for each interrogated CpG unit and allows for data export. Finally, an additional layer of data processing can be performed through the use of the MassArray bioconductor package [58] or other analytical tools where needed.

Each reaction should start with 10 ng or more of bisulfite-converted DNA. Using this amount, it has been demonstrated that a methylation level of 5% can be distinguished from a complete lack of methylation. The standard deviation of relative DNA methylation levels between 10 and 90% is approximately 5%; the measurement variance increases outside of this analytical range [49]. The ability to multiplex assays within a single well is sacrificed for the quantitative determination of multiple CpG dinucleotides, a property shared by most direct quantitative methods. While direct multiplexing is limited, the parallel assessment of multiple markers from multiple samples can be rapidly assayed. Indeed, a high-throughput MALDI-TOF is capable of processing 6000 reactions per day [49]. For example, the previously described assessment of 47 targeted regions across 96 individual samples could be performed in a single day [49]. Overall, EpiTYPER analysis provides a high-throughput method for quantitative DNA methylation analysis, lending itself to biomarker validation and development.

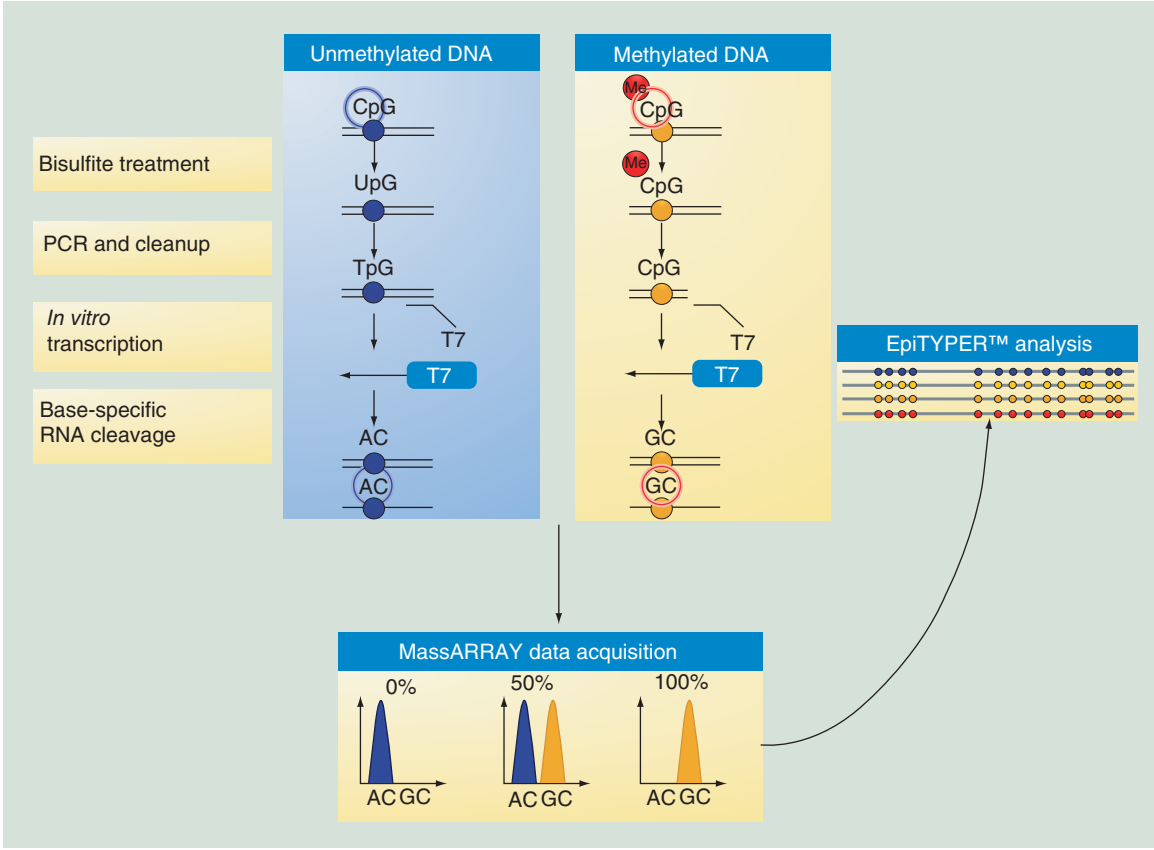

Figure 4. Schematic describing DNA methylation analysis using MassARRAY (EpiTYPER analysis). DNA is first treated with sodium bisulfite, resulting in a methylation-dependent sequence change. Target regions are subsequently amplified, products subjected to *in vitro* transcription and base-specific cleavage, using RNase A and data acquired on the MassARRAY MALDI-TOF mass spectrometer. Data are then processed and interpreted using EpiTYPER software. Colors represent DNA methylation levels (blue = unmethylated, red = methylated). Intermediate colors highlight that the DNA methylation measurement is quantitative, resulting in the ability to discern intermediate DNA methylation levels.
For color images please see online www.futuremedicine.com/doi/full/10.2217/EPI.14.43

General sample considerations

Tissue: native fresh frozen, formalin fixed paraffin embedded & alternative fixatives

Proper preservation of resected tissue is crucial for a broad range of biological studies. The tissue preservation method of choice for DNA methylation analysis is to deep freeze the tissue. However, deep freezing requires a lot of laborious equipment, particularly -80°C refrigerators with secured electrical power supply or liquid nitrogen storage. Consequently, the procedure of formalin fixation and paraffin embedding (FFPE), first described in 1893, evolved into the standard method for tissue fixation [59]. Formalin allows morphological preservation of tissue but degrades DNA by fragmentation and causes DNA-protein crosslinks at the expense of molecule integrity [60]. However, optimized and standardized fixation as well as optimized isolation protocols can help to overcome these problems. Time of formalin fixation heavily influences the sample quality. The longer the tissue is submerged in formalin, the higher is the molecule degradation. Moreover, cold fixation at 4°C reduces fragmentation of DNA [59,61]. To enhance isolation of high-quality nucleic acids, reagents partially reversing the DNA-protein crosslinks have been developed and have been included in protocols supporting nucleic acid isolation from FFPE samples.

Many advances have also been made in developing protocols for analyzing DNA methylation applicable to FFPE samples, including protocols for pyrosequencing [62], microarray-based genome-wide methylation studies [63] and qPCR based methods [23]. Nevertheless, FFPE tissue remains a tricky source for isolation of nucleic acids for proper quantitative methylation analysis.

Another negative aspect has been addressed by Hamilton *et al.*, who found altered methylation patterns within the MGMT gene caused by the formalin fixation compared with fresh frozen tissue [64]. Therefore, there is an increasing demand for alternative

fixation approaches to ease the use of fixed tissue in molecular biology. Such alternatives are already commercially available. RCL2, an alcohol-based fixation reagent, for example, does not degrade the DNA and widely preserves tissue morphology [13,65]. Other commercially available fixatives are PAXgene, Allprotect (both from Qiagen, Germany) and RNAlater (Invitrogen, UK). All of those fixatives allow the isolation of high-quality DNA comparable to DNA derived from fresh frozen samples. However there is evidence that Allprotect and RNAlater impair immunohistochemical analyses [66]. The authors are well aware that clinicians will not change their fixative because they are gentler on DNA and RNA. However alternative fixatives should be considered if the sample is foreseen for 'omics' studies to improve data quality.

Body fluids

The potential of DNA methylation markers for clinical diagnostics in body fluids has been shown already in many studies associated with various neoplastic diseases [67,68]. Body fluids can usually be obtained using noninvasive (e.g., saliva, sputum and urine) or minimally invasive (e.g., serum, plasma) methods and their extraction can also be implemented in routine diagnostic tests, representing significant benefits for patients as well as clinicians. On the other hand, analyzing DNA methylation markers in body fluids is technically challenging due to several factors. In many body fluids such as plasma, serum, cell-free saliva or urine, the total amount of DNA is relatively low, the amount of cell-free DNA (cf DNA) derived from affected cells is minimal in comparison to 'normal' DNA present in the fluid and cf DNA is usually highly fragmented [69–73].

DNA isolation protocols for body fluids must therefore combine processing of high sample volumes and/or highly sufficient extraction of small-scaled and short-sized DNA fragments, making the choice of the right isolation method a crucial step for successful biomarker detection. A broad range of different kits for nucleic acid isolation from various body fluids is commercially available as well as many different published methods trying to fulfill these demands. Table 2 summarizes exemplarily DNA isolation results from serum and/or plasma of healthy individuals in several independent studies, revealing large variations in DNA output, based not only on usage of different isolation approaches but also distinct DNA quantification methods, which makes it difficult to define a 'gold standard method' for DNA isolation of cf DNA out of body fluids. Furthermore amount and quality (fragmentation level) of cf DNA derived from body fluids are also strongly affected by pre-analytical parameters such as the chosen sampling procedure or sampling conditions, underlining also the high demand of standard operating procedures for sample pretreatment, especially for cf DNA analysis in clinical applications. Messaoudi *et al.* recently defined various parameters for optimal pre-analytical blood sample handling before cfDNA isolation based on literature data and confirmatory experiments which could be used as a first step in this direction (Box 1) [74–79].

Nevertheless highly sensitive DNA methylation detection methods able to identify low copy numbers of methylated DNA and working with limited amounts of available total DNA are essential to enable reliable detection of aberrantly methylated markers [91].

Apart from technical challenges, another important factor has to be considered: while aberrant DNA methylation markers detected in urine or sputum are site directed, markers in serum, plasma or saliva can originate from anywhere in the body. Therefore methylation markers identified in these substrates must be specific for a single or only a small group of diseases to enable the clinician to identify the site of malignancy, especially regarding diagnostic approaches.

Sample sizes

Concerning the statistical approach for sample size and power calculations, we refer to the specific literature [92]. Although no general numbers on sample size can be given due to heterogeneity of markers and samples, we however want to give some examples from our own studies such as lung cancer biomarkers deduced from native tissue (patent number: WO2010086388) obtaining almost perfect classification using eight markers and an MSREqPCR analysis (studying n = 96 samples; balanced case–control design). Candidate markers were then reconfirmed using pyrosequencing (n = 174, eight markers). To obtain a power of 0.9, sample size calculations using the methylation percentage values from the markers and 174 samples (comprising DNA samples from tissue of three groups of 24 normal lung, 60 adenocarcinomas and 90 squamous cell carcinomas) revealed a sample size of 18 biologically distinct samples per group (assuming a Type 1 error of 0.05 and a hypothesized mean twofold difference between classes).

We then set up a 37-plex MSREqPCR candidate assay and analyzed cfDNA from 194 plasma samples (unpublished data, not shown). These data were also used for sample size calculations and a sample size of 41 biological samples per group was estimated to obtain a power of 0.9 (when a Type 1 error of 0.05 and a hypothesized mean fourfold difference between classes was assumed; sample size doubled to 82 different biological samples per group if a Type 1 error of

Table 2. Comparison of DNA isolation results in various studies out of serum and/or plasma derived from healthy individuals.

Study (year)	Serum cfDNA concentration (ng/ml serum)	Plasma cfDNA Concentration (ng/ml serum)	Isolation method	Quantification method	Ref.
Xue *et al.* (2009)	1.67	–	QIAamp DNA Blood Midi Kit (Qiagen)	Real-time PCR (GAPDH gene)	[80]
	4.37 (1.49–10.25)	–	THP (Triton/Heat/ Phenol protocol)	Real-time PCR (GAPDH gene)	
Mori *et al.* (2005)	263 (±51)	–	QIAamp DNA Blood Mini Kit (Qiagen)	PicoGreen quantification assay	[81]
Gal *et al.* (2004)	63 (5–456)	–	QIAamp DNA Blood Mini Kit (Qiagen)	Real-time PCR (β-globin gene)	[82]
Wu *et al.* (2002)	57.1 (±30.6)	–	QIAamp 96 DNA Blood Kit (Qiagen)	PicoGreen DNA detection kit	[83]
Wielscher *et al.* (2011) (serum DNA yield varied with sample cohort)	(1) 11.9 (±10.9)† (2) 39.7 (±32.8)† (3) 12.2 (±9.7)†	5.8 (±5.1)	High pure template preparation kit (Roche)	PicoGreen quantification assay	[6]
Board *et al.* (2008)	24.65 (5–64)	5.07 (2.5–7.5)	QIAamp Viral Spin Kit (Qiagen)	Real-time PCR (AAT gene)	[84]
Gautschi *et al.* (2004)	12.6	1.8	QIAamp DNA Blood Mini Kit (Qiagen)	Real-time PCR (GAPDH gene)	[85]
Herrera *et al.* (2005)	–	10.6 (7.0–14.0)	QIAamp DNA Blood Mini Kit (Qiagen)	Real-time PCR, (β-actin gene)	[86]
Jung *et al.* (2004)	–	20	NucleoSpin Blood DNA Purification Kit (MN)	PicoGreen quantification assay	[87]
Deligezer *et al.* (2003)	–	44	NucleoSpin Blood DNA Purification Kit (MN)	Spectrophotometry	[88]
Stemmer *et al.* (2003)	–	3-22	KingFisher silicate magnetic beads	Picogreen reagent	[89]
Chang *et al.* (2002)	–	7	QIAamp DNA Blood Mini Kit (Qiagen)	PicoGreen™ DNA quantitation kit	[90]

†DNA yields varied depending on at which institution samples were taken

0.001 was assumed). We conducted test-wise the same 37-plex MSREqPCR assay on a different sample set of 48 serum samples and calculated a sample size of 64 different biological samples per group from that data for obtaining a power of 0.9 if a Type 1 error of 0.05 and a fourfold difference was assumed. Although we used the same methods and protocols for cfDNA isolation and methylation analyses, the sample source effected assay performance and calculated sample size (more than 50% increase in numbers when comparing the samples per group for the 'plasma-cohort' (n = 41) and 'serum-cohort' (n = 64) from our example).

Using another dataset to confirm methylation markers on DNA from native glioblastoma and normal brain tissue derived DNA [13], testing of eight candidate gene regions by Sequenom's Epityper assays made a sample size of 18 biologically distinct samples per group necessary to obtain a power of 0.9 (assuming a Type 1 error of 0.05 and a hypothesized mean twofold difference between classes). This is very similar to the number found in comparison to the pyrosequencing-based lung cancer study described above. Although sample size is very dependent on assay's performance and standardization, we still believe that the numbers from our examples should be useful for conception of pilot experiments. As can be assumed from comparing numbers from the examples of cfDNA methylation analyses from plasma and serum from different retrospective cohorts and study sites, pilot studies should be performed for distinct sample sources. Especially for cfDNA testing samples from different sources might not be useful for direct comparisons.

Overview of established markers & current methylation marker validation studies

Regarding the clinical implementation of DNA methylation biomarkers, several studies are currently testing the use of early detection biomarkers as well as of prognostic and predictive biomarkers in malignant diseases (for a more comprehensive review on DNA methylation based biomarkers and clinical implementation, see [93] and [18]). Here we want to briefly discuss kits already existing in the market, which use established markers for early detection of colon, lung and prostate cancer and for predicting recurrence of bladder cancer (Table 3).

In colon cancer, promoter methylation of septin 9 (SEPT9) and vimentin (VIM) can be used for early detection of malignant tissue by analyzing blood (SEPT9) or stool (VIM) samples of patients [94–97]. Both markers show improved sensitivity and specificity when compared with the fecal occult blood test, which is normally used as a standard noninvasive screening test for colorectal cancer.

In lung cancer, methylation of SHOX2 is used as a biomarker in clinical settings for distinguishing malignant and benign lung diseases [35,98]. SHOX2 methylation is measured in bronchial aspirates, and a sensitivity of 78% and a specificity of 96% have been reported [98].

In prostate cancer research, the latest and probably best-studied methylation marker recently implemented in the clinics is methylation of GSTP1, as it is seen in over 90% of prostate cancer patients, but not in normal prostate or benign prostatic hyperplasia [99]. The promoter methylation status of GSTP1 in urine or plasma can be evaluated as a follow-up in individuals at risk, for example, after a positive PSA (prostate-specific antigen) test, which would significantly improve specificity and reduce false-positive results after PSA screening. Likewise, a way to reduce false-negative results after histopathological classification of prostate biopsies is to determine the methylation status of GSTP1, APC and RASSF1. This is possible as promoter methylation of certain genes is also changed in the surroundings of malignant tissue, a phenomenon called 'halo effect,' and thus biopsies taken from histopathological nonmalignant areas are able to reveal the presence of adjacent malignant tissue [100].

In people with hematuria, the DNA methylation status of TWIST-1 and NID-2 is used together with other biomarkers to rule out bladder cancer, and Vimentin and NID-2 methylation form part of a panel of markers assessing the recurrence of bladder cancer [101–103].

Among the group of predictive DNA methylation biomarkers used to predict the response to chemotherapeutic drugs, many belong to the group of DNA repair genes. In line with the finding that absence of repair genes renders tumors more susceptible to alkylating agents, methylation of the MGMT gene, which encodes the DNA repair protein O6-methylguanine DNA methyltransferase, has been shown to be associated with a survival benefit of glioblastoma patients after treatment with the alkylating drug temozolomide [104,105].

Conclusion

A variety of methods are nowadays available for efficient analyzes of DNA sequences with changed methylation patterns. Usually (human/vertebrate diagnostic) methods focus on the detection of a gain in 5mC methylation, found in the CpG dinucleotide context. This makes it relatively simple to design primers and assays for bisulfite deamination based PCR amplification and DNA methylation testing. Alternatively, MSRE and MBD-affinity capture based methods for selective fractionation, amplification and quantification of methylated sequence regions are in use. However, these sequences have a high GC content and thus a high melting temperature, which can cause some difficulties in assay set-up. Methods reviewed here, are all well suited for either parallel analyses of multiple regions of interest, which might be of interest, when, for example, hundred or more methylation sites have to be confirmed upon genome-wide screening, and for efficient high-throughput analyses of many clinical samples, which is most often required for biomarker

Box 1. Guidelines for optimal pre-analytical blood sample handling before cf-DNA isolation.

- Use plasma samples instead of serum to avoid contamination of blood–cell genomic DNA
- Use Ethylenediaminetetraacetic acid or cell-free DNA collection tubes for blood sampling to prevent blood–cell lysis
- Process blood samples within 4 h after drawing blood to retain initial DNA concentration and integrity
- Include a second high-speed centrifugation step after first blood sample centrifugation to remove any remaining cells
- Aliquote plasma/serum samples to avoid freeze–thaw cycles, do not freeze/thaw samples more than two-times to preserve initial DNA integrity
- Store plasma/serum samples at -80°C and perform DNA isolation within 9 months after sampling to preserve initial DNA integrity

Table 3. Commercially available tests based on DNA methylation biomarkers.

Biomarker	Application	Disease	Material	Sensitivity/ specificity (%)†	Commercial test	Ref.
SEPT9	Early detection	Colorectal cancer	Blood	70–80/89–99	Epi proColon® 2.0 (Epigenomics), ColoVantage™ (Quest Diagnostics), Real-Time mS9 (Abbott)	[94]
VIM	Early detection	Colorectal cancer	Stool	92/87	Cologuard™ (Exact Sciences)	[96]
SHOX2	Early detection	Lung cancer	Sputum	81/95	Epi proLung® BL 1.0 (Epigenomics)	[98]
GSTP1 + APC + RASSF1	Confirm negative biopsy	Prostate Cancer	Prostate biopsy	74/63	ConfirmMDx for Prostate Cancer (MdxHealth)	[100]
GSTP1	Early detection	Prostate cancer	Urine	–	Predictive Biosciences	[99]
MGMT	Predictive	Brain cancer	Tumor	–	PredictMDx™ Brain Cancer (MDxHealth)	[105]
TWIST2 + NID2	Predictive	Bladder cancer	Urine	87.9/99.9	CertNDx™ Bladder Cancer Assay Hematuria Assessment (Predictive Biosciences)	[101]
VIM + NID2	Recurrence	Bladder cancer	Urine	90.5/95.5	CertNDx™ Bladder Cancer Assay Recurrence Monitoring (Predictive Biosciences)	[103]

†As indicated by the provider.

validation. Common to all methods is that they provide a quantitative readout of the methylation value. qPCR-based methods usually provide an overall methylation-measure of the amplified sequence, relative to a calibration curve. Pyrosequencing, deep sequencing and MALDI-based testing provide a quantitative single 5mC site-specific readout. Depending on the methylated sequence and sample type of interest for validation, methods have to be carefully qualified usually with *in vitro* methylated and unmethylated DNA.

An extremely critical issue in DNA methylation testing is the preprocessing of the analyzed samples. Along these lines we underline in this article that freshly deep-frozen specimens, which have not undergone any fixation procedure, represent the best starting material for DNA methylation analysis. We further conclude that methylation testing of cell-free DNA in body fluids is quite challenging because of the limited amounts of cf DNA compared with cell-derived DNA and underline to preferentially use deep-frozen plasma instead of serum for methylation testing in blood samples.

We further show in the present review sample-size calculations for genome-wide methylation screening studies which, independent from which platform technology is used, reveal that sample numbers of approximately 30 per group still lead to false discovery rates of 20%. Concerning sample size in validation studies, we state from own experiences that they are highly dependent on assay's performance and standardization and that especially for cf DNA testing different sources of samples should be avoided since they appear not to be useful for direct comparison.

Last but not least, methylation marker validation studies and established diagnostic DNA methylation markers for cancer have been summarized in this review. These might be the forefront of future validation studies for various diseases based on recent methylation screening initiatives.

Future perspective

DNA methylation based biomarker development has increased exponentially over the last decade. Especially in but not solely limited to oncology, DNA methylation based biomarker discovery studies have been very successful and will be best suited for diagnostic, predictive and prognostic testing. Due to the stability of the DNA as well as the methylation pattern, cfDNA methylation testing as well as a couple of tissue-based assays have found their way into the clinics. Although validation of biomarkers is challenging, an appropriate variety of different methods are available, which enable efficient design and qualification of methylation assays for validation studies. We estimate that qPCR-based assays will remain the working horse for these studies,

for the upcoming few years. Although deep amplicon bisulfite sequencing provides quantitative methylation values and a single C resolution of methylation patterns, PCR amplification is a prerequisite for deep sequencing. For most approaches, methylation quantification using PCR is sufficient and more cost-effective. We expect that introduction of third-generation sequencing omitting PCR amplification, and enabling a direct, bisulfite-

Executive summary

Methods and strategies for DNA methylation testing and validation

Quantitative DNA methylation analysis with methylation-sensitive restriction enzymes

- This type of DNA methylation analysis typically involves the use of MSRE that cut only unmethylated DNA and enables, in combination with quantitative PCR, a reliable and simple detection of DNA methylation targeting native DNA; pre-amplification protocols enable multiplexed analyzes even when testing rare fractions of methylated (tumor) DNA in cell-free DNA is aimed.
- Complete digestion of the DNA prior to PCR is absolutely essential and ensured by at least two to three cut sites within the target region and the use of a combination of different MSRE.
- The design of MSRE qPCR assays is straightforward since native DNA sequence is used for primer design; considering multiple MSRE cut sites per amplicon warrants reliable detection of hypermethylated regions. To qualify assays and to exclude experimental bias, primers should be tested prior to use as suggested by the MIQE guidelines.

Methyl-CpG-binding domain affinity capture based quantification

- MBD (Methyl-CpG binding domain) fusion proteins specifically bind to dsDNA that is methylated at CpG sites on both strands. Stringency of the binding is controlled by NaCl concentration.
- MBD affinity assays have a large dynamic range. Successful fractionation is possible with less than 0.1 ng of genomics DNA but also from formalin fixed paraffin embedded samples where sensitivity is a major issue because the majority of DNA is unamplifiable due to frequent crosslinks and strand breaks, a problem still aggravated by bisulfite treatment based methods.

Bisulfite-based quantitative PCR testing

- Two different assay setups are applied in qPCR-based methods that use bisulfite-treated DNA: methylation-independent PCR (MIP, no CpGs in primer sequence) and methylation-sensitive PCR (MSP, CpGs within primer sequence).
- MethyLight, the first quantitative MSP assay, employs MSP combined with methylation-sensitive probing (TaqMan) and is capable of detecting methylated DNA in a 10,000-fold access of unmethylated DNA. SMART-MSP, a more economic variant of MethyLight, relies on probe-free MSP using DNA intercalating fluorescent dyes combined with high-resolution melting analysis, which allows for the identification of false positives.
- The Heavy Methyl assay, which is used in the kits 'Epi proColon' and 'Epi proLung' from Epigenomics, uses blocking oligos, binding to unmethylated DNA, which overlap with primer-binding sequences, thus allowing for amplification of methylated sequences only.

Pyrosequencing-based methylation analysis

- In pyrosequencing-based methylation analysis, CpG bearing sequences of interest are amplified by PCR after bisulfite conversion utilizing flanking CpG-free regions. One of the PCR primers is biotinylated for a streptavidin bead based cleanup of the biotinlyated single strand which is finally sequenced.
- Successful pyrosequencing is heavily dependent on quality and quantity of the PCR product. Along these lines, assay design (unique software Pyromark Assay Design 2.0, Qiagen) and PCR optimization (thermal profile, the ratio of biotinylated vs nonbiotinylated primers and avoidance of primer dimers) are important.
- DNA inputs of 10–100 ng DNA per PCR reaction reliably detect DNA methylation down to 5%. Pyrosequencing-based methylation analysis is considered to be the gold standard in single-target screening approaches as well as in validation of DNA methylation microarray results.

Targeted deep amplicon bisulfite sequencing

- With the availability of next-generation sequencing technology, deep sequencing upon bisulfite conversion has become a very effective approach for analyzing methylation patterns and is currently the successor method of the previous 'bisulfite genomic sequencing' when cloning of single amplicons into plasmids and Sanger sequencing of multiple clones per amplicons has been conducted for analyses of different clones of DNA at a single CpG resolution.
- The massively parallel sequencing can easily and directly sequence pools of more than 100 bisulfite PCR products, generated by methylation-independent amplification (MIP) of many targets in single PCR reactions; analyzing different patient samples is easily achieved by barcoding pools of amplicons derived from a specific patient in a single sequencing run.

Quantitative DNA methylation analysis with MALDI-TOF MS

- In this method (Epityper analysis), subsequent to bisulfite conversion, a region-specific PCR reaction is performed to enrich the region of interest. The amplified product is then subjected to a simultaneous *in vitro* transcription and base-specific RNA cleavage with the products measured using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
- Quality of Epityper analysis is dependent on both the assay and the input material (10 ng DNA or more).
- With up to 6000 assays per day, Epityper analysis provides a high-throughput method for DNA methylation analysis where data interpretation is supported by the EpiTYPER software.

Executive summary (cont.)

General sample considerations

Tissue: native fresh frozen, formalin fixed paraffin embedded, alternative fixatives

- Freshly and deeply frozen tissue - and body fluid samples are best to use for DNA methylation analysis.
- When fixed specimens have to be used, reduction of formalin fixation times, the use of reagents partially reversing DNA–protein crosslinks as well as commercially available alternatives for formalin fixation should be considered if any possible.

Body fluids

- Cell-free DNA methylation analysis in body fluids is challenging due to the minimal amount of cfDNA compared with cell-derived DNA and the fact that cfDNA is usually highly fragmented.
- General recommendations for optimal serum/plasma preprocessing: use plasma instead of serum when possible, use EDTA or cell-free DNA collection tubes, store plasma/serum at -80°C, avoid freeze–thaw cycles.

Sample sizes

- Sample size and power calculations for validation studies depend on assay performance, effect size and experimental design. When cases versus controls (or different groups of samples, patients etc.) are to be tested, performance of standardized (methylation) assays is best determined using samples from the same source as the validation study will be conducted. As in every other experimental setup, cases and controls should have been pretreated (also prior methylation analyzes) in a similar manner.

Overview of current methylation marker validation studies and established markers

- Several studies are currently testing the use and clinical implementation of DNA methylation biomarkers including early diagnosis as well as disease prognosis and prediction.
- DNA methylation marker kits for early detection of lung, colon and prostate cancer are already in the market.

Future perspective

- DNA methylation biomarker discovery and validation studies will further increase and expand to diseases other than cancer.
- qPCR-based assays will remain investigators' first choice for validation studies.
- Third-generation sequencing methods, enabling PCR- and bisulfite-free DNA methylation analysis, should revolutionize and improve the field.

free readout of DNA methylation (and other types of modification), will unquestionably improve the field of DNA methylation analyses. It is also evident that within the next few years digital PCR testing of methylation analyzes will make its way into clinical research and diagnostics. For confirmation of multiple candidate markers using many samples, the high-throughput assays will be of certain use.

Within the next 10 years, we expect that DNA methylation based markers will be validated in suitable cohorts and make their way into the clinical routine to facilitate patient screening, monitoring and stratification for therapy decision making.

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