

# A critical appraisal of tools available for monitoring epigenetic changes in clinical samples from patients with myeloid malignancies

Kirsten Grønbaek,<sup>1</sup> Carsten Müller-Tidow,<sup>2</sup> Giovanni Perini,<sup>3</sup> Sören Lehmann,<sup>4</sup> Marianne Bach Treppendahl,<sup>1</sup> Ken Mills,<sup>5</sup> Christoph Plass,<sup>6</sup> and Brigitte Schlegelberger<sup>7</sup> on behalf of the European Genomics and Epigenomics Study on MDS and AML (EuGESMA), COST Action BM0801

<sup>1</sup>Department of Hematology, Rigshospitalet, Copenhagen, Denmark; <sup>2</sup>Department of Medicine, Hematology/Oncology, University of Münster, Germany; <sup>3</sup>Department of Biology, University of Bologna, Italy; <sup>4</sup>Department of Internal Medicine/Hematology, Karolinska University Hospital Huddinge, Stockholm, Sweden; <sup>5</sup>Centre for Cancer Research and Cell Biology, Queen's University Belfast, UK; <sup>6</sup>Department of Epigenomics and Cancer Risk Factors, German Cancer Research Center (DKFZ), Heidelberg, Germany; and <sup>7</sup>Centre for Pathology and Forensic and Genetic Medicine, Institute of Cell and Molecular Pathology, Hannover Medical School, Germany

## ABSTRACT

Research over the past decade has confirmed that epigenetic alterations act in concert with genetic lesions to deregulate gene expression in acute myeloid leukemia and myelodysplastic syndromes. Epigenetic alterations may serve as markers of disease, and may potentially be used for classification, prognostication and to monitor minimal residual disease. In addition, we now have the capability to pharmaceutically target epigenetic modifications, and there is an urgent need for early validation of the efficacy of the drugs. Also, an improved understanding of the functionality of epigenetic modifications may further pave the road towards individualized therapy. The recent advances in biotechnology and bioinformatics provide a plethora of novel tools for characterizing the epigenome in clinical samples, but at this point the practical, clinical utility of these methodologies needs further exploration. Here, we provide the pros and cons of the currently most feasible methods used for characterizing the methylome in clinical samples, and

give a brief introduction to novel approaches to sequencing that may revolutionize our abilities to characterize the genomes and epigenomes in acute myeloid leukemia and myelodysplastic syndrome patients.

**Key words:** myelodysplastic syndrome, epigenetic, alterations, monitoring, biotechnology, bioinformatics.

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## Introduction

Epigenetic alterations in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) have profound effects on gene expression and may cause tumor suppressor gene silencing. A large number of genes are inactivated by hypermethylation of CpG islands in the promoter regions. AML, and MDS in particular, have extensive aberrant DNA methylation when compared to normal CD34 positive bone marrow cells.<sup>1</sup> In addition, genome-wide promoter DNA methylation profiling reveals unique AML subgroups and methylation patterns that are associated with clinical outcome, opening up new avenues for improved diagnostics and prognostication.<sup>2</sup> Since thousands of gene promoters typically undergo DNA hypermethylation, there is a rationale for using DNA methyltransferase inhibitors (DNMTI), which are the first epigenetically targeted therapies to have shown efficacy in the treatment of MDS and AML.<sup>3-5</sup> New drugs that target different epigenetic changes, i.e. histone modifications, are under development. To offer an individualized therapy, reliable methods to monitor the efficacy of these novel therapies are needed.

Here, we summarize currently available analyses used for this purpose and highlight important issues in the choice of methodology.

### Clinical utility of DNA methylation profiling

Over the last couple of decades, many studies have addressed the use of promoter methylation status of individual genes for prognostication in myeloid malignancies. Early studies showed that promoter methylation of the *CDKN2B* (p15) gene was associated with disease progression in MDS<sup>6,7</sup> and secondary AML,<sup>8</sup> and that the *CDKN2B* promoter was demethylated during treatment with 5-aza-2'-deoxycytidine (5-aza-CdR).<sup>9</sup> However, other studies imply that the clinical importance of *CDKN2B* methylation in AML is controversial.<sup>10,11</sup> Nonetheless, methylation of different individual genes and combinations of genes (e.g. *CDH1*, *HIC1*, *SOCS1*, *BCL2L10*) were shown to have prognostic impact in MDS and AML,<sup>11-15</sup> increasing expectations that a global screening approach would improve the clinical utility of DNA methylation profiling.

At present, the majority of global methylation profiling

studies have been performed in AML, with only a few studies in MDS and chronic myeloproliferative disorders (CMPD). In AML, several studies have shown a link between global methylation profiles and distinct chromosomal rearrangements such as t(8;21), inv(16), t(15;17), MLL rearrangements or complex karyotype but, in addition, new prognostic sub-groups have been identified based only on their methylation profile.<sup>2,11,16,17</sup> Furthermore, recent studies show a correlation between increased DNA methylation and mutations in enzymes involved in DNA demethylation (*IDH1/2* and *TET2*),<sup>17,18</sup> while another study shows association of *TET2* mutations to global hypomethylation.<sup>19</sup> Whether this relates to diversities in methodology or biology is a subject for further investigation. Furthermore, global and gene-specific DNA methylation levels have been associated with the expression level of miR29b, a microRNA that targets the 3'UTR of DNMT3A and 3B, and the Sp1 promoter element of *DNMT1*, leading to their downregulation and thus hypomethylation.<sup>20</sup>

In MDS, only a few global methylation profiling studies have been published. In a screen of 807 promoters, increased methylation was associated with transformation from low- to high-risk MDS.<sup>21</sup> In a different study, a comprehensive screen of 14,000 promoters revealed that the basic level of methylation in MDS and secondary AML is much higher than in *de novo* AML and in normal CD34<sup>+</sup> cells.<sup>1</sup> However, so far, no global methylation studies have addressed the prognostic impact of global methylation changes in MDS; however, a decrease in methylation was observed after 5-azacytidine (5-aza-CR) treatment.<sup>1</sup> There have been no global methylation profiling studies in CMML published so far, and a single study of CMPDs (polycythemia vera and essential thrombocytosis) did not reveal differential methylation when compared to normal controls.<sup>22</sup>

Accordingly, only limited quantities of global methylation profiling data in myeloid malignancies are currently available, and those available are from studies based on different approaches to methylation detection. Some studies only analyze methylation at a few CpG sites at the promoters of selected cancer-associated genes,<sup>11,21</sup> while others also include promoter methylation of genes that have not previously been implicated in cancer.<sup>1,2,10,17,18,22</sup> But all of the above-mentioned studies focus on a variable number of selected CpG sites, predominantly at CpG island promoters. Since recent studies suggest that cytosine methylation outside gene promoters may also be involved in gene regulation, an unbiased approach may provide new information.<sup>23,24</sup> The current technological advances allow for a global digital mapping of CpG methylation that may potentially change our view of the role of DNA methylation in myeloid malignancies. However, at this point, the technology and data management needed is not to be underestimated. Hopefully, some of this new knowledge may translate into clinically useful tools.

### Clinical utility of histone modification profiling

Histone modifications play an important role in determining cell fate, temporal and spatial organization of transcription, DNA repair and multiple other cellular functions. In hematologic malignancies, aberrations occur in writers as well as readers of histone modifications. For example, mutations of the H3K27me3 histone methyl-

transferase Enhancer of Zeste2 (EZH2) occur in malignant myeloid disorders.<sup>25,26</sup> In addition, in myeloproliferative syndromes, the frequently mutated JAK2 kinase directly phosphorylates histones.<sup>27</sup> As will be discussed below, the analyses of histone modifications in clinical specimens are currently more difficult than the analyses of DNA methylation. Nonetheless, a few studies have been published that indicate that the profiling of histone modifications in clinical specimens is feasible.<sup>28-31</sup> These experiments were performed with ChIP-chip procedures. Importantly, recent improvements in methodology appear to make the use of primary patient samples more easily accessible. These procedures will be discussed below.

### Methodologies for detection of DNA methylation

Approaches to screen for epigenetic alterations in myeloid malignancies have mainly taken two directions. The first approach relies on the evaluation of specific candidate cancer genes, frequently representing known tumor suppressor genes.<sup>32</sup> The second strategy employs a global screen for epigenetic alterations. Although genome-wide DNA methylation analyses provide new insights into the methylation pattern of normal cells and cancer cells, there are limitations to the extent to which genome-wide techniques give full and accurate assessment of the methylation status of individual genes or CpG sites. As a result, validation of gene and/or site-specific methylation is usually recommended as a complement to genome-wide methods.

Until now, investigations have been designed to focus on promoter CpG-island hypermethylation. Recent data indicate that the methylation status of CpGs outside the classical CpG-island promoter regions also influence transcriptional regulation. Methylation at the so-called CpG-island shores,<sup>23</sup> in enhancer regions,<sup>33</sup> and non-CpG island promoters<sup>24</sup> may also influence gene expression. In addition, methylation of the repetitive LINE1 sequences has been used as a proxy marker to monitor the overall methylation level of cancer epigenomes in AML and MDS.<sup>34-36</sup>

### Methodologies for global DNA methylation profiling

Currently used methods for global DNA methylation profiling are listed in Table 1. Methods based on methylation-sensitive restriction enzymes are limited in their use due to the location of restriction sites. These approaches, as for example Restriction Landmark Genomic Scanning (RLGS)<sup>37</sup> or HpaII tiny fragment enrichment by ligation-mediated PCR (HELP),<sup>38</sup> are being replaced more and more by methods that to a lesser extent pre-select the investigated sequence. Instead, enrichment for methylated DNA molecules is being accomplished by: i) using enrichment of methylated DNA sequences by antibodies or proteins binding methylated DNAs; or ii) methods based on bisulfite conversion (Figure 1).

#### Methods based on enrichment of methylated DNA

One approach uses an anti 5-methyl cytosine (anti-5mC) antibody for methylome profiling in a technique called methylated DNA immunoprecipitation (MeDIP).<sup>39,40</sup> First, the DNA is sonicated, denatured to single-strand DNA and then incubated with the anti-5mC antibody. Alternative methods enrich for methylated DNA by binding to methyl CpG binding domain proteins (MBDs). This may be done using MBD3L1, a binding partner of MBD2

that increases the affinity of MBD2 for methylated DNA for enrichment (Methylated-CpG island recovery assay (MIRA)).<sup>41,42</sup> In an alternative approach, Methyl CpG immunoprecipitation (MCIp) was developed utilizing antibody-like recombinant protein (MBD2-Fc) and A-Sepharose columns for the enrichment of methylated DNA from genomic DNAs.<sup>43</sup> Enriched DNA can either be labeled and hybridized to whole-genome tiling DNA arrays (ChIP-on-chip) or analyzed using next generation sequencing (ChIP-seq; see below)<sup>44</sup> (Figure 1A and B). These protocols allow a comprehensive analysis of the methylation pattern across the entire genome.

#### Methods based on bisulfite conversion

At present, most of the commonly used methods for methylation detection at the single gene and whole genome levels are based on the initial treatment of the DNA template by sodium bisulfite. Sodium bisulfite converts C to uracil (U) while it leaves 5mC intact.<sup>45</sup> In the subsequent PCR reactions, U will be amplified as thymine (T), while the static and unconverted 5mC is amplified as C.

One of the most commonly used methods for global methylation detection uses the bead-based arrays from Illumina. After bisulfite treatment, the DNA is amplified, fragmented and hybridized to a large set of short oligonucleotides bound to beads. Each unique genomic region is represented by two homologous oligonucleotides that only differ by a specific C/T, and thus gives a relative measure of the amounts of mC *versus* C in the template (Figure 1C). This method has been able to record the methylation status of approximately 27,500 promoter island CpGs, but a new chip now enables the recording of more than 485,000 CpGs, including CpGs at non-CpG

islands, in non-coding regions and at miRNA promoters.<sup>46</sup> However, this is still much below the estimated number of approximately 28 million CpG dinucleotides present in the whole genome.

#### Next generation sequencing of the methylome

As for chromatin immunoprecipitated DNA, bisulfite-converted DNA may be analyzed by next generation sequencing (NGS). In this process, simultaneous amplification of hundreds of sequences from bisulfite-converted or immunoprecipitated DNA is followed by the sequencing of up to several hundred million reads. Since the value of the data is directly proportional to the number of times a particular genomic region is sequenced in parallel (so-called coverage), a complete sequencing is currently cost-prohibitive for studies of multiple samples. Despite these issues, several groups have recently published whole DNA methylome data from various hematopoietic cell types including human pluripotent and differentiated stem cells,<sup>47</sup> and peripheral blood mononuclear cells.<sup>48</sup>

Reduced Representation Bisulfite Sequencing (RRBS) is a modification of this technology. The *MspI* restriction enzyme that cuts the sequence 'CCGG' is used to enrich for genomic regions that show two of these sites within a distance of 300 bp or less. As a consequence, CpG islands and promoters are significantly enriched. The subsequent sequencing allows quantitative assessment of more than  $1 \times 10^6$  independent CpGs.<sup>49</sup> This procedure is relatively cost-effective and allows a higher degree of quantitative DNA methylation analysis than MeDIP based assays.

#### Methods for confirmation of methylation status at single gene or CpG site level

The methods reviewed below are summarized in Table

**Table 1. Methods for genome-wide methylation profiling.**

Methodology	Minimum amount of DNA	Advantages	Limitations
Restriction enzyme-based method			Dependent on distribution of restriction enzyme sites
RLGS	1.5 µg		Limited coverage of the genome Low sensitivity
HELP	10 µg	Better sensitivity in regions with low CpG island density	
Methylated DNA enrichment			Lacks full coverage of the epigenome Dependent on antibody quality
MBD protein enrichment - array	5 µg	Good coverage of both CpG islands and of repetitive elements	Low sensitivity in low density CpG regions
MBD protein enrichment - seq	3 µg	Best coverage, not dependent on array design	Is demanding on costs and bioinformatic resources
MeDIP-array Antibodies against mC/hmC	5 µg		Complicated by single stranded DIP
MeDIP-seq Antibodies against mC/hmC	3-5 µg		Complicated by single stranded DIP
Bisulfite conversion-based methods		Provides information on the individual CpG dinucleotides	
Illumina Array Chip	0.5-1 µg	Fast and easy to use in multiple samples	Detects methylation only at the selected CpG sites
Whole genome bisulfite sequencing	5 µg	High resolution and full coverage with whole genome sequencing	Is demanding on costs and resources
Reduced representation Bisulfite sequencing	0.5 µg	Quantitative analysis	Methylation analyses centered towards CpG islands

2. Most of the currently used methods for DNA methylation detection at the single gene level rely on the initial bisulfite conversion of the DNA. This will allow separation of methylated and unmethylated alleles by two major mechanisms: i) by specifically amplifying methylated and unmethylated alleles in two different PCR reactions; or ii) by a physical separation of a PCR product containing a mixture of methylated and unmethylated DNA.

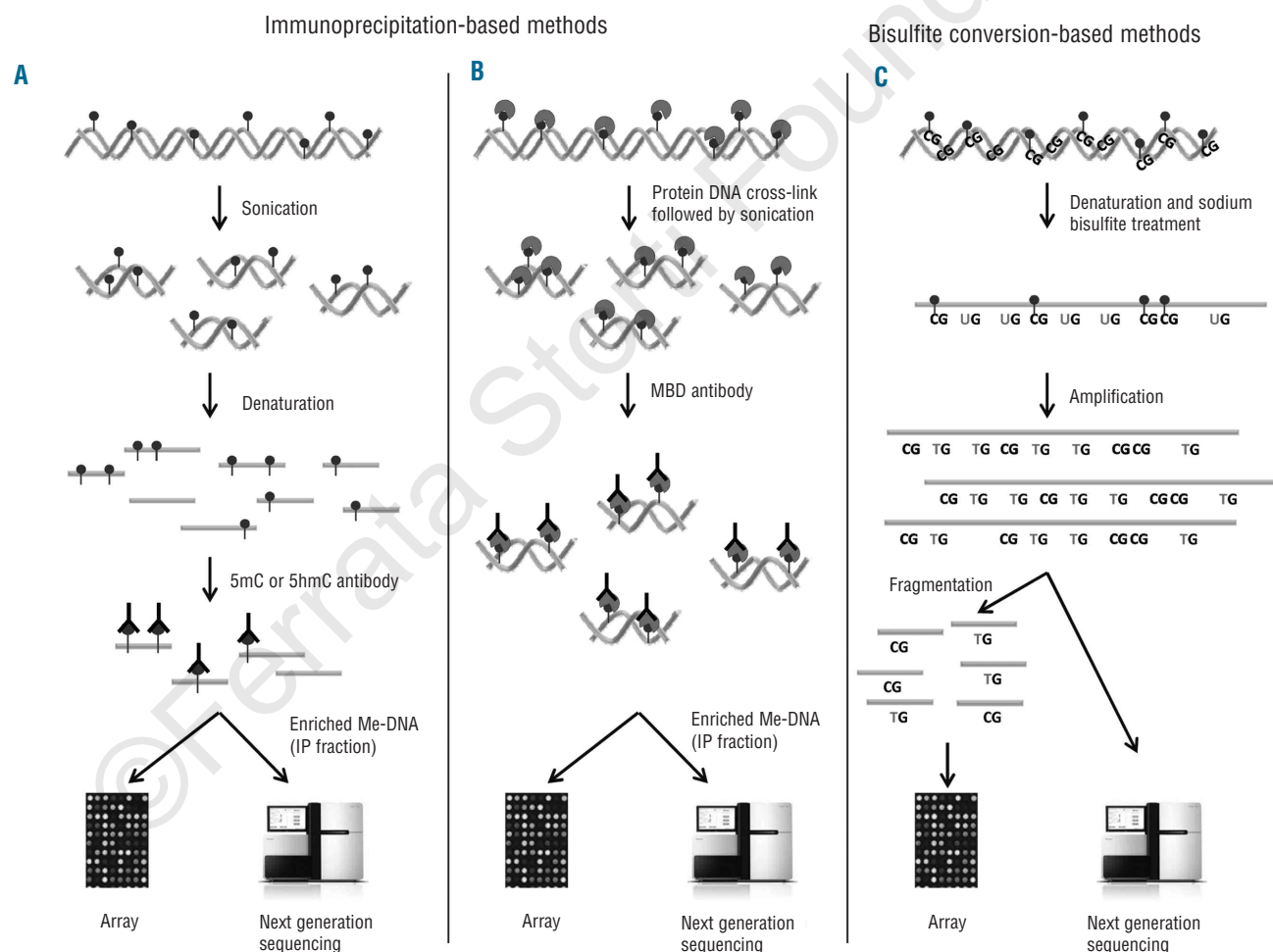
#### Screening for methylation at individual gene promoters

Methylation-specific PCR (MSP) is without doubt the most frequently used method used to detect methylation at individual genes. The advantage of MSP is the relatively simple experimental design and its high sensitivity with a detection rate of 1 methylated allele in 1,000 unmethylated alleles.<sup>50</sup> However, the major disadvantages are its rela-

tively low specificity, the fact that methylation is only investigated at the binding sites of the primers, and that it only detects methylation on a single or a few cytosine molecules.

More detailed information on the pattern and density of methylation can be achieved by methylation-specific melting curve analysis (MS-MCA). This method is based on the fact that the melting properties of bisulfite-treated methylated and unmethylated DNA species differ considerably and can, therefore, be separated by melting the different PCR products in a thermal cycler (LightCycler).<sup>51,52</sup> The advantages of these methods are their high specificity, and that they allow semi-quantification of methylation and resolution of variable methylation patterns at different molecules in the same specimen.

The MethyLight assay is a TaqMan fluorescence-based,



**Figure 1.** Methods to detect global DNA methylation. (A) Methylated DNA immunoprecipitation (MeDIP). DNA is sonicated and subsequently denatured. Single stranded DNA is incubated with antibodies against either methylated (5mC) or hydroxymethylated (5hmC) cytosines followed by IP. The IP fraction (enriched for either methylated DNA or hydroxymethylated DNA) can either be labeled and hybridized to whole-genome tiling arrays or analyzed using next generation sequencing (NGS). (B) Chromatin immunoprecipitation (ChIP) of methylated DNA. DNA and protein are cross-linked by formalin followed by sonication. DNA is incubated with antibodies or proteins that bind MBDs and precipitated. The enriched fraction can be analyzed by the same methods as for MeDIP. (C) The DNA is denatured and treated with sodium bisulfite. Unmethylated cytosine, 'C', is converted to uracil, 'U', while 5mC or 5hmC are unchanged. In the subsequent amplification, 'U' is amplified as thymine, 'T', while the unconverted 5mC or 5hmC are amplified as 'C'. The amplification products are fragmented and hybridized to bead-based arrays or analyzed by NGS.

**Table 2. Common methods for determining single gene or CpG site-specific DNA methylation**

Method	Advantages	Limitations
Methylation-specific PCR (MSP)	High sensitivity Simple design Fast	Low specificity Detects only methylation of a single or few sites
Methylation-specific melting curve analysis (MS-MCA)	Higher specificity Semi-quantitative	Relatively simple primer design Fast run
MethylLight	Very high sensitivity Possibility of assessment of MRD Quantitative	Expensive Complex primer design
Bisulfite sequencing	Gold standard Individual alleles are investigated Methylation status of each CpG site	Requires cloning Labor-intensive and expensive
Pyrosequencing	High resolution quantification of consecutive CpG sites Fast Multiple samples can be run	Limited number of consecutive CpG sites
MassARRAY	High resolution quantification of consecutive CpG sites or CpG units Fast Multiple samples can be run	Resolution of single CpG dinucleotides is sometimes not possible (average methylation data are given for CpG units)

real-time PCR method, which gives a quantitative measure of the amount of methylated alleles in a mixture with unmethylated alleles. The average level of methylated CpG sites in the amplified region is calculated; however, methylation at individual CpGs is not resolved. The advantage of this method is its high sensitivity that allows for detection of one methylated allele among 10,000 unmethylated alleles. Accordingly, MethylLight is efficient in situations where a limited number of methylated alleles are detected in a mixture with abundant wild-type DNA, e.g. in the detection of minimal disease in body fluids such as sputum, serum and urine. Also, it gives a quantitative measurement of the amount of methylated alleles. The disadvantages are the cost and the relative complexity of the assay.

#### Quantitative analysis of methylation at individual CpG sites

To obtain detailed information about the methylation status at every single cytosine in a particular sequence, bisulfite genomic sequencing has long been the gold standard. However, methylation detection by pyrosequencing has recently been accepted as a robust alternative.

In bisulfite genomic sequencing, the bisulfite-converted DNA sequence is cloned into a plasmid vector in order to obtain substantial amounts of template for direct sequencing. In this way, individual alleles are examined for their specific methylation pattern, and the relative methylation density of individual cytosines can be estimated.<sup>45</sup> The method is rather laborious, and requires the sequencing of at least 10 clones per transcript in order to obtain reliable results.

By contrast, the pyrosequencing technology allows direct, high-resolution quantification of methylation density over several consecutive CpG sites. The method is based on the release of pyrophosphate (PPi, P<sub>2</sub>O<sub>7</sub><sup>4-</sup>) during nucleotide extension.<sup>53</sup> Typically, protocols recommend that the sequence analyzed should be no longer than 300 bp; however, in reality, much is resolved by analyzing sequences no longer than approximately 100 bp.

The advantages of this method are that it is fast and allows for the detection of 96 samples in a single run, but in contrast to bisulfite sequencing, methylation is not resolved at individual alleles.

In the MassARRAY assay, bisulfite-converted DNAs are amplified with PCR primers that do not discriminate between methylated or unmethylated sequences. The resulting PCR product is transcribed into RNA, which is subsequently cleaved by RNase A. The resulting fragments are then quantitatively tested for their DNA methylation status using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.<sup>54</sup>

#### Current challenges and novel approaches to DNA methylation detection

##### Methods that distinguish 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC)

The discovery that the frequently mutated TET2 proteins can convert 5mC to 5hmC has challenged the use of bisulfite-based methods for DNA methylation detection.<sup>55,56</sup> Recent data suggest that TET proteins survey DNA methylation fidelity at transcription start sites of TET target genes,<sup>57</sup> but at present the abundance and distribution of 5hmC in MDS and AML, and its exact role in transcriptional regulation, is still a question of debate. However, it has clearly been shown that bisulfite conversion does not distinguish 5mC and 5hmC, and that 5hmC containing regions are poorly amplified by PCR after bisulfite conversion.<sup>58</sup> In order to discriminate these two functionally different species, MeDIP-based technologies may be applied. DNA immunoprecipitation by antibodies specific to 5hmC and 5mC are followed by PCR for detection of methylation at the single gene level, and by whole genome sequencing or array analysis for global methylation detection. A major obstacle has been the specificity of the antibodies that discriminate 5mC and 5hmC, respectively, but recently developed alternative methods using enzymatic and chemical steps to isolate a few or single molecule hmCs may improve separation of the two species.<sup>59</sup>

### 'Third generation' sequencing of methylated nucleotides

In the near future, it is envisioned that the cost of sequencing will be further reduced and the sequencing of multiple cancer genomes is undertaken in international consortia. Multiple reads per sequence are required due to the heterogeneity of prior DNA methylation patterns representing a certain sequence. Initial bisulfite treatment or ChIP would no longer be required as single molecules such as 5mC and 5hmC can now be detected directly from the sequencing reads. Novel technologies, such as single molecule real-time (SMRT) sequencing,<sup>60</sup> single-molecule nanopore sequencing with protein pores,<sup>61</sup> or 'Ion-chip' technology that directly senses the ions released during DNA synthesis<sup>62</sup> may replace current technologies. However, for the moment, further documentation of their power and specificity for methylation detection in clinical samples is needed.

### Analyses of histone modifications

Posttranslational modifications of the core histones constitute an important part of the epigenetic memory.<sup>63</sup> Each of the core histones is strongly conserved throughout phylogenesis and can be modified at multiple residues primarily on its N-terminal tails by multiple enzymes.

#### Chromatin-IP analysis to identify histone modifications

Histone modification analyses in AML have been fuelled by the development of ChIP as a suitable method to identify posttranslational histone modifications at specific gene promoters. Initially introduced in yeast, this method has matured over the last ten years and is currently the standard approach.<sup>64</sup> ChIP coupled with real-time PCR allows the identification of histone modification patterns of single promoters and enhancers. The possibility of covering a region of several kilobases with interspaced amplicons has enabled researchers to clearly define binding patterns of transcription factors and of regions of histone modifications with high sensitivity and specificity.<sup>65</sup> Nonetheless, this approach still requires an *a priori* hypothesis of relevant genes and loci.

The development of unbiased methods, such as ChIP-chip and ChIP-seq, has vastly improved the potential of chromatin IP-based analyses of histone modifications.<sup>66</sup> The basic principle underlying ChIP-chip and ChIP-seq is very similar: sheared chromatin with specific histone modifications is enriched by antibody-based precipitation. The DNA is subsequently isolated. For ChIP-chip analyses, the usually very small amount of DNA needs to be amplified and labeled. Finally, a hybridization step onto specific microarrays is needed. In contrast, for ChIP-seq, libraries can be generated from small amounts of DNA available after ChIP and these can be sequenced using NGS. Nonetheless, there are several critical points that are common to both procedures. First of all, the antibody used for ChIP is of vital importance. Specificity of the antibody needs to be rigorously controlled. For the ChIP procedure itself, it is necessary to have smaller fragments of chromatin with DNA between 400 and 1,000 base pairs long. The widely used sonication bears the problem that intensity needs to be adjusted for each cell type. It is also possible to use enzymatic digestion and this can be performed by several methods. Another important problem and critical point in the analyses of histone modifications in leukemia samples relates to the controls used. One control for changes in leukemia is made up of CD34<sup>+</sup> progenitor

cells, but in specific instances it may be feasible to use a cell population that more closely resembles the transformed cells as control.

A general problem is the variety of histone modifications found *in vivo* and their potential crosstalk. Given that two alleles are usually present, it might be difficult to assess whether the histone modifications shown as more or less well defined peaks are found on the same chromosome or on different chromosomes within the same cell. Sequential ChIP has been described as one solution to this problem.<sup>66,67</sup> In this method, the immunoprecipitated histones are used for a subsequent ChIP to ensure that the resulting sample is enriched for both modifications on the same histones.

Standard chromatin IP methods require high numbers of cells as starting material. In contrast, most clinical specimens have a rather limited number of cells available. An important issue is, therefore, to identify the minimum number of cells that are required for a ChIP-seq experiment. In the last few years, several procedures have been developed that make use of a limited number of available cells. In 2007, Attema *et al.* used about 50,000 purified stem cells for H3K4 methylation and acetylation analyses.<sup>68</sup> Recently, protocols developed by Bradley Bernstein's group have developed protocols (so-called nano-ChIP-seq) in which cell numbers as low as 10,000 can be successfully used.<sup>69-71</sup> These protocols contain significant changes in the chromatin IP procedure itself but especially also in the library preparation. Typically, such protocols also involve a separate amplification step to obtain sufficient DNA.

In addition to genome-wide histone modification analyses with identified loci, it is also possible to quantify total histone modification levels. One recent approach used mass spectrometry-based methods.<sup>72</sup> Due to the abundance of histones in normal cells, mass spectrometry can be used to quantify and identify the posttranslational modification patterns.<sup>73,74</sup> This approach might be especially interesting for control of epigenetic therapy whereby global alterations of histone modifications can be analyzed. Since ChIP-seq and ChIP-chip analyses are unlikely to be involved in diagnostic procedures in the near future, it might also be interesting to look at approaches to identify histone modification changes at a few specific loci. One possibility here is to couple a chromatin IP with subsequent PCR amplification. However, problems with standardization and the amount of work involved in this procedure make such approaches currently unsuitable for use with clinical specimens on a larger scale. Technologies such as nano-string might help to improve throughput and reproducibility.<sup>75,76</sup> This technology is based on complementary sequence-specific probes coupled to biotin and molecular tags that can be easily identified and quantified. It is possible that the coupling of this nano-string technology to chromatin IP material might help to improve quantification.

An important problem for ChIP-chip and ChIP-seq relates to the bioinformatic analyses of the data. Excellent reviews have been published on this topic.<sup>77</sup> The bioinformatic analyses are currently being further improved, especially for ChIP-seq.

#### Future developments in histone analysis

The rise of NGS has provided novel opportunities to decipher epigenetic changes. With the further increase in sequencing capacity most groups now focus on ChIP-seq

of histone modifications in leukemia. But several points remain to be clarified. Of note, histone modifications as analyzed by ChIP-based techniques leads to relative enrichment data only. This is in contrast to DNA methylation studies including methods that allow determination of the exact methylation status of each CpG dinucleotide. It is increasingly clear that the histone code is very complex and that the same histone modification might have a different impact in different regions of the genome. Finally, only the integration of histone modification pattern data, along with transcription factor binding studies, RNA-expression and large genome sequencing studies will reveal the true relevance of these patterns for leukemogenesis.

### Technical requirements of methods used to monitor epigenetic therapy

Since technologies for epigenetic monitoring are constantly evolving, the recommendations for sample handling and requirements may change rapidly. However, for the time being, a few issues may be worth considering.

#### Optimal handling of patient material for epigenetic profiling

The correct selection and handling of patient material is crucial, since the generated data are only as good as the input material allows. Although new approaches imply that, for example, formalin-fixed paraffin-embedded (FFPE) tissue may be used for genome-wide analyses, it is recommended to use fresh or fresh frozen material in order to get as few artifacts as possible. For bone marrow samples with a high fraction of AML blasts, density centrifugation could be used for further enrichment of blasts, which might be sufficient to define global DNA methylation patterns and to compare the patterns of a particular histone modification. In cases in which specific subpopulations need to be analyzed, it might be worthwhile to use magnetic cell sorting or FACS sorting. For MDS samples, sorting of the malignant clone might be necessary, but often difficult. However, using granulocytic fraction may lead to an enrichment of malignant cells and clearer results.<sup>78</sup>

Any method that relies only on the use of DNA is particularly attractive in a clinical setting due to its stability that, for example, allows overnight shipment at room temperature. In contrast, methods involving ChIP typically require careful and relatively fast handling of material, and the cells must be analyzed fresh or fresh frozen. In addition, some analyses may be optimized by immediate crosslinking of the DNA and chromatin of fresh cells. Another issue is the amount of material needed which is typically larger in ChIP-based approaches. However, novel methods are being developed that allow ChIP experiments with smaller amounts of patient material, as described above. Requirements for the individual analyses are listed in Table 1.

### Monitoring the effects of epigenetic therapy

Since the responses to epigenetic therapy are only observed well after treatment has started, there is a relentless demand to find markers that allow determination of treatment efficacy at an early time point. This has been investigated for 5-aza-CdR<sup>9,13</sup> for combination treatment of 5-aza-CR and the selective Class I HDACI Entinostat,<sup>1,79</sup> and for 5-aza-CR and valproic acid (VPA).<sup>80,81</sup> These studies clearly show that DNA methylation can be reversed by epigenetic therapy at an early time point during treatment, and that this alteration persists onto the next treatment cycle.<sup>1,9</sup> In neither of the studies could the baseline level of methylation be used to predict response to epigenetic therapy. However, in the 5-aza-CdR study, reduced methylation levels over time during treatment could predict response. In this study, the methylation level of 10 selected genes was measured quantitatively by pyrosequencing. In the 5-aza-CR/entinostat trial, neither gene re-expression, nor promoter methylation changes of 4 selected genes, as measured by MSP after the first treatment cycle, could predict response after 4 cycles.<sup>79</sup> These observations may indicate that quantitative measurements of methylation may be crucial for the development of markers that can select patients upfront who will benefit from treatment with DNMTI. Another explanation might be that the effects of 5-aza-CdR are more directly linked to DNA hypomethylation, whereas methylation-independent effects, such as RNA damage, may play a larger role in 5-aza-CR mediated responses.<sup>82</sup> However, in the combined 5-aza-CR/VPA trial, responders showed a relative demethylation and increased expression of the cell cycle regulator PI-PLCβ1 over time,<sup>81</sup> and a recent study suggests that *BCL2L10* methylation may be a useful prognostic marker for outcome in patients treated with 5-aza-CR with and without the addition of VPA.<sup>14</sup>

Finally, a special issue relates to the material used when monitoring the effects of epigenetic therapy; in the responders the malignant cells may have completely disappeared and molecular investigations are then performed on the residual healthy bone marrow. Thus, surrogate markers in normal tissue may be even more efficient in monitoring effects of epigenetic therapy. Obviously, these investigations should ideally be performed in large, prospective clinical trials.

### Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at [www.haematologica.org](http://www.haematologica.org).*

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