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PII: S1382-6689(17)30035-2
DOI: http://dx.doi.org/doi:10.1016/j.etap.2017.02.004
Reference: ENVTOX 2710

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 25-10-2016
Revised date: 2-2-2017
Accepted date: 3-2-2017

Please cite this article as: Tapia-Orozco, Natalia, Santiago-Toledo, Gerardo, Barrón, Valeria, Espinosa-García, Ana María, García-García, José Antonio, García-Arrazola, Roeb, Environmental epigenomics: Current approaches to assess epigenetic effects of endocrine disrupting compounds (EDC's) on human health.Environmental Toxicology and Pharmacology http://dx.doi.org/10.1016/j.etap.2017.02.004

Environmental Epigenomics: current approaches to assess epigenetic effects of endocrine disrupting compounds (EDC's) on human health

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Abstract

Environmental Epigenomics is a developing field to study the epigenetic effect on human health from exposure to environmental factors. Endocrine disrupting chemicals have been detected primarily in pharmaceutical drugs, personal care products, food additives, and food containers. Exposure to endocrine-disrupting chemicals (EDCs) has been associated with a high incidence and prevalence of many endocrine-related disorders in humans. Nevertheless, further evidence is needed to establish a correlation between exposure to EDC and human disorders.

Conventional detection of EDCs is based on chemical structure and concentration sample analysis. However, substantial evidence has emerged, suggesting that cell exposure to EDCs leads to epigenetic changes, independently of its chemical structure with non-monotonic low-dose responses.

Consequently, a paradigm shift in toxicology assessment of EDCs is proposed based on a comprehensive review of analytical techniques used to evaluate the epigenetic effects. Fundamental insights reported elsewhere are compared in order to establish DNA methylation analysis as a viable method for assessing endocrine disruptors beyond the conventional study approach of chemical structure and concentration analysis.

Keywords: Endocrine-disrupting chemicals, monitoring, epigenetic analysis

1. Introduction

Pharmaceuticals, personal care products, food additives, and plastics, among others, are considered essential to modern lifestyle. Nevertheless, many of these chemical compounds have been found in unexpected regions. For example, the antiviral medication Tamiflu was found in surface waters in United Kingdom (Singer *et al.*, 2007); 17 micropollutants (e.g., bisphenol A (BPA), diclofenac, naproxen, di-2-ethylhexylphthalate (DEHP), triclosan, etc.) were determined in surface and ground waters in Mexico City (Félix-Cañedo, 2013); and even illicit drugs have been found at selected locations of the Colorado Basin in the United States (Jones-Lepp *et al.*, 2012). The toxicity data of these molecules are not completely available or have not yet been fully investigated. Thus, assessing the impact of these molecules on human health has become a pressing need for global agencies and institutes aimed at protecting public health and environment care, such as the World Health Organization (WHO) and the European Commission (Falconer *et al.*, 2006; Hutchinson *et al.*, 2013; Mc Clellan & Halden, 2010).

Some contaminants with particular chemical structures and properties that interfere with endocrine systems have been of increasing interest. These endocrine-disrupting chemicals (EDCs) are poorly regulated, and information on their environmental impact is currently incomplete (Campbell *et al.*, 2006; Kundakovic & Champagne, 2011; Petrovic *et al.*, 2004). However, preliminary results from *in vivo* models, and epidemiological and clinical studies, have shown that EDCs play a role in diseases such as breast cancer and prostate cancer; metabolic diseases such as obesity; and effects on the human reproductive, thyroid, cardiovascular, and neuroendocrinology systems. Therefore, it has become a global public

health issue (Anway *et al.*, 2005: Diamanti-Kandarakis *et al.*, 2009; Gore *et al.*, 2011; Kundakovic & Champagne, 2011; Li *et al.*, 1997; Mc Lachlan, 2001; Svechnikov *et al.*, 2010).

Besides wastewaters, other sources of EDCs related to human activity are present, for example, in high-volume horizontal hydraulic fracturing. This new mining technique is employed for shale gas extraction, using chemicals known to exert neurotoxic, carcinogenic, and endocrine-disrupting effects. The economic implications of natural gas extraction have resulted in insufficient investigation into the health implications (Rafferty & Limonik, 2013). Furthermore, the information related to nanoparticles and carbon nanotubes has raised in the last years (Iavicoli *et al.*, 2013; Lam *et al.*, 2006; Lanone *et al.*, 2013; Lu *et al.*, 2013; Nel *et al.*, 2006).

Sampling and identification of EDCs in environmental matrices is expensive and time consuming. In this regard, the US Environmental Protection Agency (EPA) has proposed various methods for EDC screening, considering factors such as persistence, bioaccumulation potential, and toxicity (PBT approach). Nevertheless, this strategy has been criticized as it overlooks chemical usage rates and real-world biodegradability (Venkatesan & Halden, 2014).

Identification of novel substances such as EDCs should be based on their long-term effects in organisms and the environment beyond the conventional accumulation, distribution, metabolism, or excretion analysis. Epigenetics can lead to a gene expression change in response to ambient factors and can inherit potential adverse alterations underlying the

genome, as reported by Guerrero-Bosagna *et al*., (2013). Therefore, environmental matrices can be measured in terms of general epigenetic activity. Many of the mechanisms by which EDCs exert their genotypic and phenotypic effects remain unknown, but there is emerging evidence related to epigenetic deregulation. Consequently, efforts related to toxicology and safety assessment have focused on epigenetic mechanisms related to exposure to chemical compounds *in vitro* and *in vivo* (Greally, 2011). In this review, we suggest environmental monitoring of EDCs by measuring their epigenetic effects through the analysis of DNA methylation.

2. Epigenetics and mechanisms of EDCs

In 1942, Conrad H. Waddington introduced the epigenetics term and described the multiple ways in which epigenetics can regulate gene expression, resulting in a particular phenotype. In fact, epigenetics research includes a variety of events, such as messenger RNA (mRNA) silencing through microRNAs (miRNAs), chromatin remodeling, histone modifications, and DNA methylation. Histone modification and DNA methylation are heritable events, but they do not involve DNA changes or mutations (Jaenisch & Bird, 2003).

Hormones are known to be signaling molecules capable of modifying gene expression in non-mammalian and mammalian vertebrates. The changes in gene expressions could be determined by DNA methylation and chromatin modifications (Andres *et al.*, 1984; Anway *et al.*, 2005; Edinger *et al.*, 2013). Cellular imprinting by EDCs may be caused by two mechanisms: the EDC may imprint the gene either through a process leading to genetic

change (e.g., DNA methylation) or by altering key steps in cell differentiation signaling pathways such that gene expression could form a biochemical memory (McLachlan, 2001). In order to understand the process by which EDCs interact and regulate gene expression, it is important to note that genes are not expressed alone but rather in the context of other genes and their products, cells, and tissues in a temporal/spatial dimension (Crews & McLachlan, 2006). If an external stimulus like an emerging contaminant alters endogenous hormone secretion or sensitivity of tissues, then alternative development pathways could be present due to these endocrine interactions (Nijhout, 2003).

EDCs affect the anatomical development and histological organization of male and female reproductive structures, resulting in fertility issues, as well as cancer of the female reproductive tract. Therefore, it is logical to assume that EDCs act by interacting with the respective sex hormone receptors (Bernal & Jirtle, 2010; Gore *et al*., 2011; Guerrero-Bosagna *et al.*, 2013; Knower *et al.*, 2014; Svechnikov *et al.*, 2010; Uzumcu *et al.*, 2012). The interaction of EDCs with sex hormone receptors can trigger signaling pathways that generate epigenetic changes in DNA methylation, which may be heritable, resulting in gene expression changes without DNA sequence mutations (Alworth, 2002; Anway *et al.*, 2005; Kundakovic & Champagne, 2011; Li *et al.*, 1997). Epigenetic changes involved in cell differentiation in affected tissues are common, which were first demonstrated by Li *et al*. in 1997. The authors discovered that upon exposure to a synthetic estrogen, diethylstilbestrol (DES), female mice showed an altered pattern of CpG methylation in the lactotransferrin gene promoter region in the uterus. Specifically, an abnormal demethylation of a CpG island occurs in response to neonatal DES exposure and adult ovarian hormones. These experiments led to the hypothesis that DES, and other environmental estrogens, can alter

the estrogen-mediated differentiation of reproductive tract target cells, that is, an epigenetic mechanism. Furthermore, in gestating female rats, Anway *et al.* (2005) demonstrated that transient exposure to EDCs such as vinclozolin (antiandrogenic compound) or methoxychlor (estrogenic compound) ~~of~~ during ~~the~~ gonadal sex determination period induced a decrease in spermatozoid viability and cell number in the first-generation adult phenotype, thus male infertility increase. The decrease in spermatogenic production was inherited through four subsequent male generations. Moreover, abnormalities during pregnancy such as anemia, preeclampsia, and blood cell defects, were observed. Although, sexual differentiation effects and infertility have been correlated with DNA methylation changes, no evidence of the role of specific genes in these events was reported. Nevertheless, the whole genome effect has been elucidated. Epigenomic changes caused by EDCs might lead to transgenerational inheritance due to their wide distribution and the stability of target cells (Greally, 2011).

Other epigenetic mechanisms involve molecular regulators such as histone variant, histone post-translational modifications, nucleosome positioning chromosome looping, DNA structural variations, and RNA-mediated regulation (Beiter *et al.*, 2009; Bernstein *et al.*, 2007; Gibney & Nolan, 2010; Hartley & Madhani, 2009; Hiragrami-Hamada *et al.*, 2009; Jia *et al.*, 2007; Klose & Bird, 2006). These mechanisms are closely related to chromatin conformation and, therefore, polymerase accessibility for gene expression. Studies have suggested an ordered pathway for chromatin architecture conformation, because nucleosome positioning requires histone deposition and sequences such as nucleosome-free regions (NFRs) (Hartley & Madhani, 2009).

Regarding genetic regulation mediated by histone modifications, Hiragami-Hamada *et al*. (2009) demonstrated that gene silencing was associated with histone H3 trimethylation at lysine 9 (H3K9me3) in an animal model. They also showed that histone H3 acetylation at lysine 4 and di- or tri-lysine methylation (H3K4me2/3) were very common modifications related to changes in gene expression. These and other histone modifications were associated with transcription regulation; however, no known histone code was related to the regulation processes mediated by hormones, and neither were these modifications associated with EDCs.

The study of miRNAs as epigenetic regulators of gene expression has expanded significantly in recent decades, although it remains a developing area, because a single miRNA can have several target mRNAs and can be regulated by different miRNAs (Klein *et al*., 2005; Kosik, 2006; Zhang & Ho, 2014). Several predictive software to determine putative targets of different miRNAs are available, but the generation rate of false positives is still high; thus, experimental evidence is necessary (Wang & Wang, 2006). Experimental findings have shown a relationship between miRNA activity and the respective targets. However, cells show different types of epigenetic regulation depending on the cell environment and stimulus received. In addition, it has been observed that the regulation process is related to tissue and is time specific, so different phenotypes are produced (Fraga *et al.*, 2005; Greally, 2011; Thompson *et al.*, 2010).

Further work is needed in order to establish regulation marks in different genomic contexts, since its understanding remains elusive. Fortunately, considerable insights have been gained and, thus far, techniques improved especially in whole-genome methylation that describes the association of epigenetic changes with exposure to EDCs in cells.

In this review, we focused on DNA methylation, since manipulation of methylation patterns is often lethal. Moreover, findings regarding maladaptive traits confirm the relevance of DNA methylation in human biological development as well as other living organisms (Crews, 2009).

3. Analysis of epigenetic mechanisms through DNA methylation

The epigenetic and epigenome regulation has been elucidated through the genes implicated in endocrine signaling such as DNA methylation (Fig. 1) (Zhang & Ho, 2014). Although several epigenetic regulators are present, most studies usually focus on cytosine methylation assays, which can be quantitative strand-specific, and allow nucleotide resolution (Suzuki *et al.*, 2010); also, could involve methylation on a locus-specific or genome-wide scale (Shen & Waterland, 2007). Almost all techniques require DNA pretreatment for enrichment of methylated DNA. These approaches are based on restriction enzymes (REs), bisulfite conversion, and affinity enrichment, and some combinations thereof (Laird, 2010).

RE assays use methylation-dependent REs and are currently the most commonly used methods for epigenetic analysis. The coverage and resolution depend on the recognition sequences throughout the genome. However, RE assays can only analyze CpGs sites within the RE action range; thus, incomplete digestion may cause false positives. Differential methylation hybridization (DMH) is an example of an epigenomic analytical technique based on RE assays. In DMH, genomic DNA is fragmented with a methylation-

independent RE. Then, these fragments are ligated with adaptors. Next, the DNA is digested with the methylation-sensitive enzyme BstUI; ~~it is~~ then, it is PCR-amplified, labeled, and co-hybridized to CpG island microarrays (Yan *et al.*, 2009).

Bisulfite conversion is an accurate and reproducible technique that takes advantage of the deamination capacity of unmethylated cytosines by sodium bisulfite, and its later conversion to uracil or thymine at higher rates than methylated cytosines. This method represents the gold standard for detecting changes in DNA methylation due to a nucleotide-level resolution and detection of methyl-specific single-stranded DNA (ssDNA) as a conversion or simple overtreatment. Some sequencing methods based on bisulfite conversion include bisulfite sequencing (BS), pyrosequencing, and combined bisulfite restriction analysis (COBRA).

In the BS method, bisulfite-treated DNA is PCR-amplified with methylation-independent primers (methylation-specific PCR or MSP) and size-fractionated via gel electrophoresis (Darst *et al.*, 2010; Herman *et al.*, 1996). The products purified by PCR were cloned into *Escherichia coli*, and five to ten individual clones were sequenced. The Infinium Methylation Assay is a commercial example of this approach (Dedeurwaerder *et al.*, 2011). COBRA is a variation of BS, and it combines bisulfite conversion, amplification PCR, and RE digestion with BstUI (Xiong & Laird, 1997). In the pyrosequencing method, bisulfite-modified DNA is amplified with the enzyme DNA polymerase and then sequenced with specific primers. During the formation of the complementary DNA strand, pyrophosphate (PPi) is released, forming adenosine triphosphate (ATP), which supplies the energy to produce a luciferase–luciferin–adenosine monophosphate (AMP) complex. In the presence

of oxygen, this complex showed a proportional light signal to ATP concentration and, consequently, to PPi. (Colella *et al.*, 2003; Tost *et al.*, 2003). Some companies have developed assays for pyrosequencing such as PyroMark CpG Assays (England & Pettersson, 2005).

Assays involving affinity for enrichment methylated regions are based on the use of specific antibodies against 5-methylcytosine (5meC) to recover ssDNA, or the use of methyl-binding proteins to enrich methylated DNA. As this technique does not require RE digestion or bisulfite conversion, it does not provide information at the nucleotide level. Moreover, it requires large quantities of input genomic DNA and intensive labor, which can limit the processing of large number of samples. However, low quantities of input DNA can be recovered by amplification methods. The efficiency of coverage and resolution depends on the genomic distribution of potential affinity targets (methyl-cytosine density) and subsequent approaches (array composition or sequencing platform). Methylated DNA immunoprecipitation (MeDIP) and Methylated CpG Island Recovery assay (MIRA) are based on enrichment assays. In MeDIP, the DNA is sheared through sonication, denatured, and immunoprecipitated with an antibody against 5meC. Then, the methylated DNA is analyzed by microarrays or sequencing (Weber *et al.*, 2005). MIRA assays involve the digestion of DNA with either MseI enzyme or sonication. Then, the sheared DNA is bound to an adaptor and incubated with MBD2b/MBD3L1 proteins. Finally, the enriched DNA is amplified and analyzed by sequencing or microarrays (Rauch & Pfeifer, 2005).

Sequence-based analysis is more flexible and powerful as it allows the analysis of allele-specific DNA methylation and requires less input DNA. Epigenetic strategies for locus-

specific methylation analysis includes a wide range of assays, for example, BS (BSPP, bisulfite padlock probes), methylation-specific PCR (MSP), amplification of intermethylated sites (AIMS), COBRA, MeDIP-PCR, methylation-sensitive melting curve analysis (MS-MCA), methylation-sensitive high-resolution melting (MS-HRM), methylation-specific fluorescent amplicon generation (MS-FLAG), sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP), methylation-sensitive arbitrarily primed PCR (MS-AP-PCR), and combination of methylated DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS). New technologies have transformed small or individual loci studies into global or genome-wide studies.

Genome-wide epigenetic studies include microarrays and sequencing technologies, which are actively used for high-resolution mapping of the chromatin structure and DNA methylation (Boyle *et al.*, 2008). To study a candidate gene, targeting their promoter is usually the simplest approach. Candidate genes are mostly selected for their function, phenotype effect, or differential expression compared to a control group (Levenson & Malmikov, 2012). The advantages of this approach are less time and cost; moreover, it is a quantitative assay providing comprehensive and unbiased insights (Greally, 2011).

Of all epigenetic modifications, DNA methylation is most widely studied, due to its heritable nature, stability, and ease of measurement. Consequently, the majority of studies on the epigenetic effects of EDCs focus on these modifications. Table 1 describes some of the techniques already mentioned, as well as their respective advantages and limitations. Many of these analytical techniques involve high-cost instrumentation, highly trained operators, a time-consuming detection process, and complex pretreatment steps.

Environmental epigenomics is an emerging field, and its main goal is developing rapid detection technologies and accurate epigenome-wide assessment methods (Jirtle & Skinner, 2007). Further work is needed to evaluate the epigenetic effects of exposure to EDCs. In particular, genome-wide assays will play an important role in findings of methylation patterns, profiles, or levels, including imprinted genes and their relationship with emerging pollutants in the environment. Platform development and cost reduction of genomic technologies can promote further exploration of induced epigenetic alterations due to EDCs. Experimental data should be analyzed and integrated into biological systems through computational epigenomics to understand epigenetic regulatory mechanisms. In addition, the insights obtained can lead to novel approaches for EDC detection and environmental monitoring, including valid analytical methodologies and portable devices such as biosensors.

4. Conclusions

Chemical compounds found in pharmaceutical drugs, personal care products, food additives, and food containers interfere with endocrine systems since are involved in diseases such as prostate and breast cancer, metabolic diseases, and effects on the human reproductive, thyroid, cardiovascular, and neuroendocrinology systems. These endocrine-disrupting chemicals (EDCs) are not wide regulated, and the documentation of their environmental effects is poor.

Identification, detection and treatment of novel EDCs should be founded on their long-term effects in organisms as well as the environmental impact beyond the conventional analysis of accumulation, distribution, metabolism, or excretion. In this regard, environmental

matrices can be measured in terms of general epigenetic activity due to a gene expression changes occurs in response to ambient factors and can inherit potential adverse alterations underlying the genome. The changes in gene expressions could be determined by DNA methylation and chromatin modifications.

The epigenetic and epigenome regulation has been studied through the genes involved in endocrine signaling and their relationship with emerging pollutants in the environment. DNA methylation is the most studied, due to its heritable nature, stability, and ease of measurement. Analytical techniques are based on restriction enzymes (REs), bisulfite conversion, and affinity enrichment.

Data science as well as cost reduction of genomic technologies will stimulate the understanding of the EDCs role on epigenetic regulatory mechanisms. In addition, the insights obtained could lead to novel approaches for EDC detection and environmental monitoring, including valid analytical methodologies and portable devices such as biosensors.

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Figure 1. Conversion reaction of DNA unmethylated and methylated by bisulfite.

A) Conversion chemistry. Bisulfite conversion is the most widely used technique for studying DNA methylation; the reaction occurs under harsh conditions (low pH/high temperature).

B) Bisulfite conversion. Only unmethylated cytosines are converted to uracil and subsequently to thymine during polymerase chain reaction (PCR) amplification. Methylated and unmethylated cytosines can therefore be detected by comparing bisulfite-converted DNA to original untreated genomic DNA (Adapted from Kristensen & Hansen, 2009)

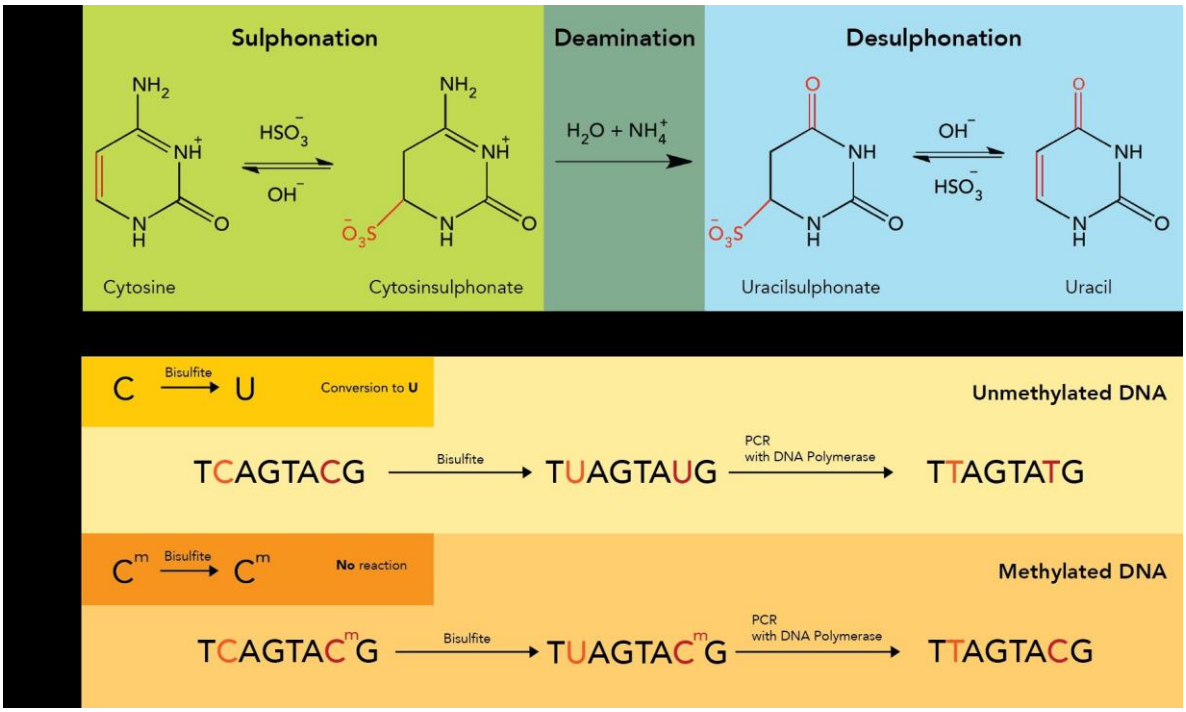

Table 1. Comparison of analytical techniques used to evaluate the epigenetic effects of EDCs.

Technique	Description	DNA Treatment	Advantage	Limitations	Reference
Differential Methylation Hybridization (DMH)	- Methylation-independent restriction enzyme that undergoes adaptor ligation. - DNA digested with methylation-sensitive enzyme *Bst*UI. - PCR-amplified. - Labeled system. - Co-hybridized to CpG island microarrays.	Restriction enzyme	- High representation of CpG islands as they contain cleavage sites. - High-throughput for site-specific detection - Gene precise	- Does not allow discrimination for allele-specific methylation. - Low representation of CpG non-enriched genomic regions. - Depends on represented microarray sequences.	Yan *et al.*, (2009); Alworth, (2002)
Methylation-specific PCR (MSP)	- DNA modification by sodium bisulfite. - Reaction of cytosines to uracil only in unmethylated regions. - Amplification of methylated and unmethylated DNA with specific primers.	Bisulfite conversion	- Assessment of CpG site methylation status within CpG island. - Requires small quantities of DNA. - Sensitive to 0.1% methylated alleles of CpG island locus - Eliminates false-positive results inherent to RE-based PCR.	- Distinction of methylated from unmethylated cytosine is dependent on sodium bisulfite treatment. - Requires optimization (lack sensitivity at low reaction temperatures). - Requires an appropriate negative control. - Not a quantitative method.	Herman *et al.*, (1996); Anway *et al.*, (2005); Dolinoy *et al.*, (2007)

Technique	Description	DNA Treatment	Advantage	Limitations	Reference
Ligation-mediated PCR (LM-PCR)	- Methylated DNA is cleaved with enzymes or chemicals. - A gene-specific oligonucleotide primer is used for primer extension, - Linker ligation and PCR are performed.	Restriction enzyme or methylation-specific strategy (e.g., DMA–piperidine)	- High-magnitude whole-genome amplification tool. - Enables amplification of PCR fragments irrespective of the genomic sequence. - Pretreatment step for MIRA, MeDIP, and sequencing. - Restriction endonuclease accessibility data from chromatin templates in limited quantity.	- Inefficient using blunt ends in the adaptor – ligation. - Potentially causes bias towards GC-poor regions. - Relatively short stretch of sequence (usually <200 bp) that can be analyzed per reaction. - Multiple steps required.	Mueller and Wold, (1989); Edinger *et al.*, (2013)
Methylated DNA Immuno-precipitation (MeDIP)	- DNA sequential treatment: sonication, denaturation, and immunoprecipitation (antibody against 5-methylcystidine). - Methylated DNA is analyzed via sequencing or microarrays.	Affinity enrichment	- Sensibility to methylation high density. - Provides genome-wide methylation maps. - Allows tissue-specific differentially methylated region evaluation.	- Depending on shearing size resolution and antibody quality and specificity. - Enrichment efficiency significantly lower in regions with low CpG content. - DNA needs to be single stranded (difficult for regions of high CpG).	Weber *et al.*, (2005); Guerrero-Bosagna *et al.*, (2013)
Bisulfite Sequencing	- PCR amplification of bisulfite-treated DNA. - Fractioning by gel electrophoresis.	Bisulfite conversion	- Quantitative DNA methylation analysis. - Single CpG resolution. - Detection of strand-specific methylation. - Whole-genome approach.	- Labor intensive. - Depends on nested PCR primers to amplify the fragment of interest. - Difficult for DNA with suboptimal integrity.	Darst *et al.*, (2010)

Technique	Description	DNA Treatment	Advantage	Limitations	Reference
Pyrosequencing	- Bisulfite modification of DNA - Amplification with DNA polymerase and sequencing primers. - Formation of luciferase–luciferin–AMP complex by release of ATP: light signal is proportional to the amount of available ATP and thus PPi.	Bisulfite conversion	- High-throughput for site-specific detection - Accurate and high resolution for methylation variable positions. - Methylation in most types of repetitive sequences.	- High instrumentation costs. - Limitation by the length of the sequence read and number of CpGs analyzed in one reaction.	Colella *et al.*, (2003); Tost *et al.*, (2003); England & Pettersson (2005); Gore *et al.*, (2011)