integrity Research Journals

Journal of Animal Science and Veterinary Medicine
Volume 7(5), pages 150-159, October 2022
Article Number: AC1069AD1
ISSN: 2536-7099
https://doi.org/10.31248/JASVM2022.331
https://integrityresjournals.org/journal/JASVM

Review article

Overview of methodology for studying DNA methylation: A review

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Received 20th July 2022; Accepted 29th September 2022

ABSTRACT: DNA methylation is the addition of a methyl group to the 5th carbon atom of cytosine in a CpG dinucleotide. It is a mechanism that causes a change in expression without altering the base sequence. It is associated with a wide range of biological processes, including deactivation of chromosome X, genomic imprinting, stem cell differentiation, gene expression control, and chromosomal stability. There are several methods available to determine the methylation status of DNA samples in the field of epigenetics. However, selecting the method that is best suited to answering a particular biological question still proves to be a difficult task. This review aims to provide biologists, with an outline of methods available, for the determination of DNA Methylation, principally those new to the field of epigenetics, with a modest procedure to help guide them in the selection of the most suitable assay to meet their research requirements, but with a particular focus on commercially available tools or other simple and straightforward explanations that have proven to be effective.

Keywords: DNA Methylation, gene expression, genomic imprinting, stem cell differentiation.

INTRODUCTION

DNA methylation is a critical determinant of many biological and cellular processes, including embryonic development (Reik *et al*., 2003), X chromosome inactivation (Heard and Disteche 2006), genomic imprinting and dosage compensation (Spahn and Barlow 2003), and genome defence from molecular parasites (Kim *et al*., 2009). It is the addition of a methyl group to the 5th carbon atom of cytosine in a CpG dinucleotide. It has profound developmental and genetic consequences, yet reversible, heritable, epigenetic changes though it has the potential of altering gene expression (Jones and Gonzalgo, 1997). The methylation reaction itself involves the flipping of the target cytosine out of the intact double helix though mechanistically complex and the transfer of the methyl group from *S*-adenosylmethionine can occur in a cleft in the enzyme (Klimasauskas *et al*., 1994).

DNA methylation at the 5th carbon atom of cytosine in a CpG dinucleotide position of cytosine contributes to the epigenetic regulation of nuclear gene expression and genome stability (Robertson, 2005; Slotkin and Martienssen, 2007). Recent discoveries indicate that levels of DNA methylation in an individual is a dynamic outcome, strongly influenced by the diet during germ cell formation, embryogenesis, and post-birth exposures. DNA methylation during developmental stages may result in the loss or gain of DNA, while at any later stage may lead to increased predisposition to various diseases and aberrations (Dhar *et al*., 2021). In DNA methylation, the covalent changes in cytosine are one of the most widely studied changes in the field of epigenetics and provided a

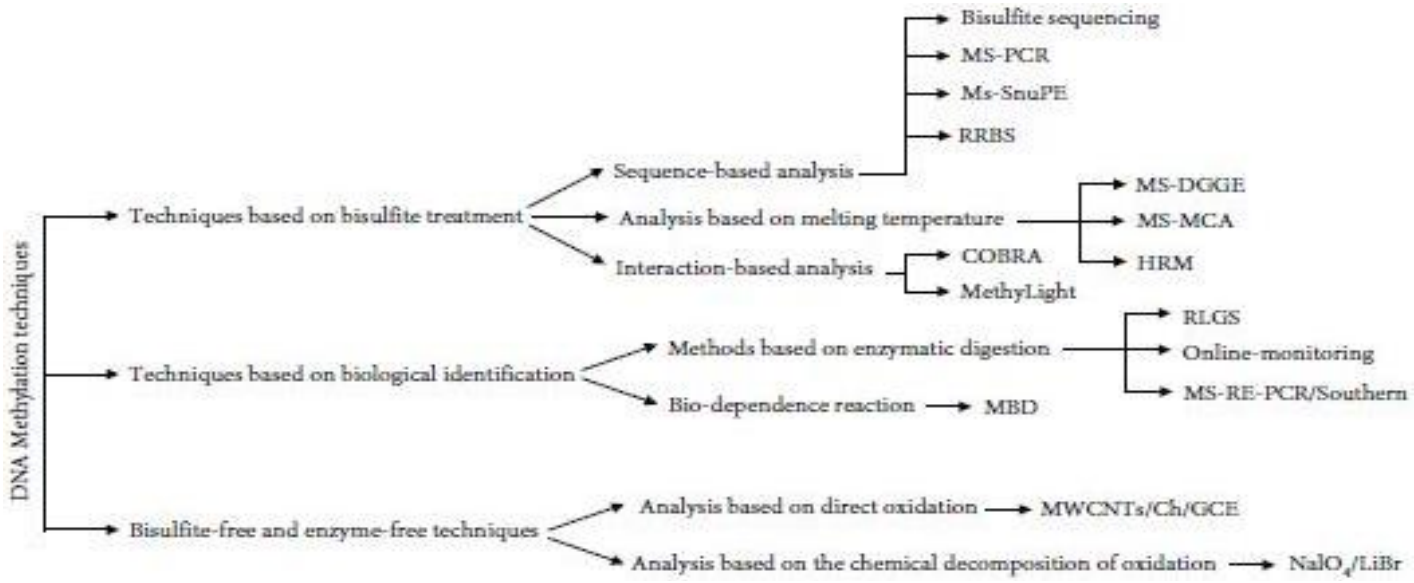

Figure 1. Schematic diagram of DNA methylation methods (Khodadadi *et al*., 2021).

molecular mechanism through which the expression of the gene can be regulated (Teschendorff and Relton, 2018; Suzuki and Bird, 2008).

In vertebrates, DNA methylation is characterized by the addition of a methyl or hydroxymethyl group to the C5 position of cytosine, which occurs primarily in the perspective of CG dinucleotides. Non-CpG methylation in a CHH and CHG context (where H = A, C, or T) exists in embryonic stem cells (Kurdyukov and Bullock, 2016). It is characterized by a wide range of biological processes, including deactivation of chromosome X, genomic imprinting, stem cell differentiation, gene expression control, and chromosomal stability (Nazor *et al*., 2012). During developmental phases, the DNA methylation pattern in the genome undergoes alterations as a result of the regulated balance between de novo DNA methylation and demethylation. Research findings show that differentiated cells receive an exclusive DNA methylation pattern that fine-tunes tissue-specific gene expression (Dhar *et al*., 2021). Further investigation into DNA methylation will lead to the discovery of new epigenetic targets, which in turn, may be useful in developing new therapeutic or prognostic research tools for diseases such as cancer that are characterized by abnormally methylated DNA (Lu *et al*., 2006; Feinberg and Tycko, 2004; Karpiński *et al*., 2008; Maekawa and Watanabe, 2007).

Hence, the review outline the various methods, for DNA methylation (Figure 1), their significance, recent advances, and their importance in the field of epigenetics for biologists.

SIGNIFICANCE OF DNA METHYLATION

DNA methylation is important in many biological processes and disruption of DNA methylation can lead to developmental abnormalities in plants and mammals, such as failure in tomato fruit ripening and embryo lethality in mice (Robertson, 2005; Cortellino *et al*., 2011; Lang *et al*., 2017). DNA methylation is now considered to be an important molecular mechanism in a number of biological processes including genomic imprinting, tissue-specific gene expression, and possibly trans-generational effects (Razin and Ceder, 1991; Li *et al*., 1993). DNA methylation, in combination with histone modifications and non-histone proteins, defines chromatin structure and accessibility. Due to its importance, it therefore helps to regulate gene expression, transposon silencing, chromosome interactions and trait inheritance. As one of the most important modifications, DNA methylation plays an essential role in regulating the growth of the cells and their proliferation (Ehsan *et al*., 2021).

Given its importance in early development and ageing, DNA methylation is a crucial epigenetic modification to profile. The discovery of DNA methylation patterns is a fast-advancing area of investigation, which promises the possibility of methylation profiling to differentiate various tumour and cancer types, and possibly their response to chemotherapeutic agents. The Discovery of aberrancies of DNA methylation seems to be one of the most important tests in early cancer diagnosis. These significant findings regarding DNA methylation would not have been possible without the development of various profiling methods (Ehsan *et al*., 2021). Indeed, there is growing evidence that methylation plays a fundamental role in developmental processes such as genomic imprinting and stabilization of X-chromosome inactivation (Jones and Gonzalgo, 1997).

DNA methylation is required for proper gene regulation during development and in differentiated tissues and has

clinical relevance. DNA methylation is also involved in the allele-specific silencing of imprinted genes (Paulsen and Ferguson-Smith, 2001). Global analysis of DNA methylation can be performed using the pyrosequencing-based analysis of methylation patterns in repetitive elements such as ALU or LINE1 elements (Yang *et al*., 2004). While LINE1 elements do have a relatively conserved sequence allowing thus the design of a sequence-specific pyrosequencing assay for DNA methylation analysis, methylation of ALU elements is assessed by a cyclic dispensation. These assays have been widely used for the measurement of global DNA methylation changes in response to environmental stimuli (Cortessis *et al*., 2012).

DNA methylation is necessary for normal embryonic development and has numerous important functions such as gene regulation, cell differentiation control, chromatin modification, mutation accumulation, silencing of endogenous retroviruses, chromosomal integrity, and genomic imprinting control (Daniel *et al*., 2006). The importance of DNA methylation in bacteria involves protecting the bacterial genome from the invasion of extracellular DNA. There are an estimated 10^{31} viruses on Earth and most of these are the phages that infect bacteria (Breitbart and Rohwer, 2005).

There are 3 important effects of DNA methylation on the genome in the development of disease mechanisms: 1) mutational burden of 5-methylcytosine, 2) epigenetic effects of promoter methylation on gene transcription, and 3) potential gene activation and induction of chromosomal instability by DNA hypomethylation (Gonzalgo *et al*., 2005). Epigenetics was linked to a variety of diseases in humans, such as developmental diseases, autoimmunity disorders, neuropsychiatric disorders, pediatric syndromes, and cancer (Rodenhiser and Mann, 2006; Hirst and Marra, 2009).

METHODS OF DETERMINING DNA METHYLATION

Altered patterns of DNA methylation are associated with altered patterns of gene transcription, which are associated with, and may be causal for some, diseases. (Cooper and Youssoufian, 1988; Wilson *et al*., 2007; Taby and Issa, 2010; Esteller, 2007). In addition, there is growing evidence that DNA methylation patterns change during aging. (Mathers, 2006; Bjornsson *et al*., 2008; Maegawa *et al*., 2010; Rakyan *et al*., 2010; Teschendorff *et al*., 2010). Therefore, the study of these changes is becoming widespread and several laboratory techniques have been developed to examine specific DNA regions (or genes) and to carry out a genome-wide analysis. Many techniques are now available for characterizing the extent of DNA methylation (Esteller, 2007).

A very sensitive as well as specific detection of the 5mC methylation pattern on the DNA obtained from the questioned sample(s) is required to be devised to be followed routinely in a forensic laboratory. Although one of the easiest methods to detect and identify DNA methylation pattern is through the use of Illumina Infinium Bead Chip array (the latest technology is the Methylation EPIC Bead Chip (Moran *et al*., 2016) which is nonetheless a very expensive method to be used routinely in forensic analysis, however, there are other molecular approaches and techniques which have been known for decades and some of them developed recently for detection and analysis of 5mC methylation pattern. It should be noted that all these methods and analyses are based on either bisulfite treatment of DNA or the use of methylation-sensitive restriction enzyme(s) or the use of an antibody against the methylated base or a combination of them. These methods and techniques require a certain amount of DNA in picogram (pg i.e. 10-12 g) or nanogram (ng i.e. 10-9 g) or microgram (µg i.e. 10-6 g), have different resolving power of methylation status in terms of bp and some are quite expensive and others relatively less expensive. Some of these methods of determining DNA Methylation include:

Methylated DNA immunoprecipitation PCR/sequencing (MeDIP-PCR/seq)

The identification of DNA methylation patterns is a common procedure in the study of epigenetics, as methylation is known to have significant effects on gene expression, and is involved with normal development as well as disease (Beck and Rakyan, 2008; Lu *et al*., 2006; Zilberman and Henikoff, 2007; Feinberg and Tycko, 2004). Methylated DNA immunoprecipitation (MeDIP) is an efficient technique for the extraction of methylated DNA from a sample of interest (Weber *et al*., 2007; Weber *et al*., 2005; Wilson *et al*., 2006). Methylated DNA Immunoprecipitation (MeDIP) involves pulling down methylated DNA regions of the genome using an antibody raised 5mC (Borgel *et al*., 2012; Mohn *et al*., 2009). The principle of MeDIP is that genomic DNA is randomly sonicated and immunoprecipitated with a monoclonal antibody directed against 5mC (Weber *et al*., 2005). Conventionally a Dot blot method (Clement and Benhattar, 2005) can be performed with the direct addition of a 5mC antibody on the fragmented DNA immobilized on a Nylon membrane and the intensity measurement of fluorescent secondary antibody provides the information on the presence, absence, or amount of methyl groups roughly present in a given DNA sample (Koziol *et al*., 2016). Therefore, it also appears that the accuracy of MeDIP measurements decreases in regions that are very CpG-poor. The classical MeDIP protocol was originally designed to work with relatively large amounts of DNA (at least 2µg) (Mohn *et al*., 2009). MeDIP is an efficient technique for the extraction of methylated DNA from forensic human samples which can include blood, bone, and hair samples commonly used as exhibits. DNA immunoprecipitation combined with next-generation sequencing methods termed MeDIPseq can be used for

the generation of methylomes from tissue or cells using 160-300 ng of starting DNA (Taiwo *et al*., 2012). Subsequently, MeDIP was combined with a next-generation sequencing platform to investigate the DNA methylome in precious samples, such as bone marrow cells, mammalian oocytes, primordial germ cells, and preimplantation embryos. The MeDIP-seq protocol has been optimized to reach as low as 50 ng of DNA for starting materials (Taiwo *et al*., 2012).

Briefly, the genomic DNA is sonicated to obtain fragments (200-800 bp) and immunoprecipitated with monoclonal antibodies raised against 5-methylcytidine (much like methyl capture protein in the MBD-seq method). In the Methylated DNA Immunoprecipitation (MeDIP) method, total genomic DNA is sheared into random fragments, which are then immunoprecipitated with an antibody that recognizes 5-methyl-cytidine (Weber *et al*., 2007; Weber *et al*., 2005).

Antibody-Methylated DNA complex is then purified using paramagnetic beads that isolate it from the rest of non-5mC methylated/unspecific DNA. The methylated DNA so obtained can be used for MSP, methylation-sensitive restriction enzyme analysis, or genome-wide 5mC analysis through sequencing/microarray (Borgel *et al*., 2012). In the genomic analysis of methylated DNA, the fragments of 36-50 bp or 400 bp, as per the method used, with methylated reads are produced. The starting sample, called Input, contains both methylated and unmethylated fragments, while the MeDIP fraction contains mainly methylated fragments. After the immunoprecipitation reaction, the Input and the MeDIP fraction can be labeled with different dyes and co-hybridized as a two-colour experiment to microarrays of (usually) CpG-rich genomic regions to obtain an estimate of a genome-wide methylation pattern (Lisanti *et al*., 2012).

Using a visual browser such as Ensembl, these methylated reads (sequences) are aligned and compared to the human genome using alignment software such as MeQA (http://life.tongji.edu.cn/meqa/) (Huang *et al*., 2012). MeDIP-PCRs targets methylated genomic loci with starting genomic DNA as low as ~1 ng (Zhao *et al*., 2014) which could be highly useful for methylation profiling of challenging DNA samples in forensics. Borgel *et al*. (2012) performed MeDIP followed by whole-genome amplification and microarray hybridization on early-stage mouse embryos starting with 150 ng of DNA. Affinity-enrichment methods such as MeDIP were initially combined with microarray technology to compare the genome-wide DNA methylation among samples (Weber *et al*., 2005).

Methyl-CpG Binding Domain protein sequencing (MBDseq)

Methylation at CpG dinucleotides in genomic DNA is a fundamental epigenetic mechanism of gene expression control in vertebrates (Bird, 1992; Singal and Ginder, 1999; El-Osta and Wolffe, 2001). Effects of DNA methylation are mediated through proteins that bind to symmetrically methylated CpGs. Such proteins contain a specific domain, the methyl-CpG-binding domain (MBD) which consists of ~70 residues in an α/β-sandwich fold built of three to four β-twisted sheets and a helix with a characteristic hairpin loop in the opposite layer (Ohki *et al*., 1999; Wakefield *et al*., 1999; Ballestar and Wolffe 2001; Nan et al., 1993). Methyl-CpG binding domain protein sequencing (MBDseq) is another technique that is used to determine genome-wide 5mC methylation patterns of humans (Aberg *et al*., 2012). The MBD family proteins are critical players in determining the transcriptional state of the epigenome. As transcriptional repressors, MBD proteins play a major role in coordinating crosstalk between DNA methylation, histone modification, and chromatin organization to achieve a coherent transcriptional program. (Du *et al*., 2010).

Proteins with a methyl-CpG-binding domain (MBD) can bind to single methylated CpGs and most of them are involved in transcription control (Roloff *et al*., 2003). MeCP2, the first MBD-containing protein to be discovered, contains the core 70-amino acid MBD and is also characterized by the presence of a TRD (Meehan *et al*., 1992). Currently, the MBD protein family consists of eleven known proteins that contain an MBD domain. The methyl-CpG-binding protein 2 (MeCP2) was the first MBD-containing protein discovered, and subsequently, MBDs 1–6 were identified through sequence homology to the MeCP2 MBD domain (Hendrich and Bird, 1998; Baymaz *et al*., 2014).

In the MBD-seq method, genomic DNA is fragmented and the methylated sequences are pulled down by a 5mC binding protein or simply using Methyl-Miner Methylated DNA Enrichment Kit (Invitrogen) (Harris *et al*., 2010). These methylated fragments are sequenced using high-throughput sequencing techniques (Lan *et al*., 2011; Rauch and Pfeifer, 2005), and the exact position of sequenced tags is determined by comparing them to the human genome. The technique is quite effective for the measurement of the methylation status of CpG islands containing a high density of CpG sites (Fraga *et al*., 2003).

The HELP methylation assay

The HELP (HpaII tiny fragment enrichment by Ligationmediated PCR) assay is another technique used for determining the dynamic nature of methylation status of DNA from different cells/tissues or from the same cells/tissues kept under different conditions (Oda et al., 2009). The HELP assay interrogates cytosine methylation status on a genomic scale (Khulan *et al.,* 2006), comparative isoschizomer profiling of cytosine methylation. Gene level, as well as genomic level of DNA methylation, is ascertained through this technique. It employs two restriction enzymes; HpaII, methylation-

sensitive, and MspImethylation insensitive (isoschizomer), to digest the genomic DNA, contrast the digestion products generated by these enzymes, and only works well for DNA fragments with a size of 200 – 2000bp; such fragments are known as HTFs (HpaII Tiny Fragments) (Khulan *et al*., 2006). HpaII specifically digests unmethylated 5'-CCGG-3' sites and enriches the methylation deficient regions of the genome. 5mC methylation state at each locus point is determined by comparing the representations made by HpaII as well as MspI (Shaknovich *et al*., 2010). In common with other assays, HELP also allows two different samples to be compared ("intergenomic"), looking for differences in methylation between cell types (Greally, 2018).

The HELP-based assays represent examples of the use of methylation-sensitive restriction enzymes. The advantage offered by comparative isoschizomer profiling is the use of the methylation-insensitive representation for comparison, which allows much better accuracy for both microarrays (Khulan *et al*., 2006; Oda *et al*., 2009; Suzuki *et al*., 2010) and massively parallel sequencing-based (Suzuki *et al*., 2010) versions of the assay. The signal at a given locus from a HpaII representation can be influenced not only by the methylation status of that locus, but also by the size of the fragment, its base composition (both variables influencing PCR amplification), and whether the locus is mutated in any way (copy number, mutations of the CG-containing and therefore highly mutable restriction enzyme target site) (Greally, 2018). The degree of difficulty associated with performing these molecular assays is generally outweighed significantly by the challenges associated with their analysis (Greally, 2018).

Bisulfite sequencing and methylation-specific PCR (MSP)

The first step in almost all protocols for studying DNA methylation is bisulfite conversion of the DNA sequence of interest. Bisulfite conversion occurs through a number of chemical reactions (e.g., sulfonation, deamination, and desulfonation) on the DNA that transforms non-methylated cytosines into uracils (Hernández *et al*., 2013). MSP (methylation-specific PCR), can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island, without depending on the use of methylation-sensitive restriction enzymes (Herman *et al*., 1996). The treatment of DNA with sodium bisulfite (Na+HSO3) converts unmethylated cytosines to uracils, while methylated cytosines are not affected (Darst *et al*., 2010). Methylated cytosine does not undergo bisulfite reaction due to steric hindrance exhibited by the methyl group. By performing a simple PCR reaction and sequencing of the DNA, methylated and unmethylated cytosines of the DNA sequence are detected and determined.

Methylation-specific PCR (MSP) is the most convenient method for studying the methylation status of promoter regions of individual genes (Herman *et al*., 1996). Methylation Specific PCR (MSP) is a post-bisulfite treatment technique which discriminately amplifies and detects a region of interest that mostly remains methylated using methylated-specific primers (Herman *et al*., 1996). Although the technique is considered outdated by some authors, its cost-effectiveness sensitivity, and rapid deployment in the laboratory make MSP the method of choice in single gene methylation studies. DNA is fragmented (~200-800 bp) by sonication and is treated with bisulfite at lower pH (pH 5) which adds sulfite group to the C6 of cytosine, which is then followed by incubating the samples at higher pH, which removes sulfite group generating uracil. The chemical modification of cytosine to uracil by bisulfite treatment has provided another method for the study of DNA methylation that avoids the use of restriction enzymes (Frommer *et al*., 1992). In this reaction, all cytosines are converted to uracil, but those that are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine (Wang *et al*., 1980). This altered DNA can then be amplified and sequenced, providing detailed information within the amplified region of the methylation status of all CpG sites (Frommer *et al*., 1992).

An advanced method called bisulfite pyrosequencing is a quantitative method used to determine the methylation status of individual CpG cytosines from PCR amplified products using a unique sequencing-by-synthesis (SBS) method (Tost and Gut, 2007**b**) and can be used to distinguish various human body fluids. MSP is based on prior bisulfite modification of the DNA sample. After the treatment, unmethylated cytosines are converted to uracils, while 5-methylcytosines remain unaltered; thus as result, DNA strands are no longer complementary to each other. The release of pyrophosphate (PPi) from the bisulfite-treated DNA is proportional to the incorporation of dNTPs, which is converted into ATP to aid in subsequent conversions of luciferin to oxyluciferin. The MSP method may also employ primer sets for unmethylated versions of the same sequence. MSP requires only small quantities of DNA, is sensitive to 0.1% methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. MSP eliminates the false positive results inherent to previous PCR-based approaches which relied on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA (Herman *et al*., 1996). The later are useful as a control and sometimes to collect the positive data depending on the experiment. A related modified method called MethyLight MSP provides a quantitative analysis using quantitative real-time PCR (Eads *et al*., 2000), where methylated-specific primers containing fluorescence reporter anneals to the region of interest.

Further on, bisulfite modified DNA is subjected to PCR amplification using two primer pairs, of which one primer pair recognizes methylated, and another pair recognizes

unmethylated alleles. An additional methodology which distinguishes MSP-generated DNA containing a low level of methylation is high resolution melting curve analysis (HRMA or Mc-MSP) (Karpiński *et al*., 2008; Wojdacz and Dobrovic, 2007), which measures the quantitative ratio of methylated and unmethylated product as differing peaks so produced in the melting curve analysis.

As a result, well-optimized PCR reaction will provide detection of a single methylated allele among one thousand unmethylated ones. High sensitivity of the reaction enables potential application of MSP-based methods for diagnostic purposes (Zhang *et al*., 2012; Delpu *et al*., 2013). The quantitative portrait of the methylation profile for the amplicon in question can be used to distinguish many body fluids from its DNA obtained as low as ~50 pg (Vidaki *et al*., 2016). However, optimization of MSP reaction can be quite challenging, therefore, primer design is an essential step. There are numerous parameters that must be considered prior to and during the MSP primer design. This assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA (Herman *et al*., 1996).

Pyrosequencing

While Sanger sequencing has been the “gold standard” for the identification of sequence variants for a long time, pyrosequencing with its improved ability for quantification, decreased limit of detection and accelerated workflow leading to a shorter time to results, has become a valuable alternative notably for many clinical and diagnostic applications. Methods for methylation analysis may focus on profiling the whole epigenome, identifying differentially methylated regions or examining specific genes of interest (Redshaw *et al*., 2014; Singer, 2019). PCR-based methods using sodium bisulfite-treated DNA are extensively used for assessing methylation at single loci. Such is the case of pyrosequencing. Pyrosequencing is an absolute method that provides a quantitative measure of DNA methylation levels at single CpG resolution, determined from the intensity ratio of T and C, the results of which are accurate and reliable for the analysis of short DNA stretches (usually<150 bp) (Tost and Gut, 2007a).

Pyrosequencing is a sequencing method used for quantitative methylation analysis of bisulfite converted DNA. For its relative simplicity, speed and comparable results, pyrosequencing can be preferred to cloning (Frommer *et al*., 1992), a method used as a gold standard for the identification of allele specific methylation patterns (Reed *et al*., 2010).

It is a method of DNA sequencing that differs from Sanger sequencing, in that it relies on the detection of pyrophosphate release and the generation of light on nucleotide incorporation, rather than chain termination with dideoxynucleotides. It is a sequencing-by-synthesis method, where nucleotides are incorporated complementary to a template strand leading to the release of pyrophosphate (PPi) that will – after several enzymatic reactions – produce a light signal proportional to the amount of incorporated nucleotide. It is a technique that uses a sequencing-by-synthesis system which is designed to quantify single-nucleotide polymorphisms (SNPs). This method of DNA sequencing detects light emitted during the sequential addition of nucleotides during the synthesis of a complementary strand of DNA. Another advantage of pyrosequencing is that it is suitable for both CpG poor and CpG rich regions. Main drawback of this method is that only shorter regions (maximum 350 bp) can be analyzed. However, this disadvantage can be overcome by using more sequencing primers on one amplicon or by a serial pyrosequencing (Tost *et al*., 2006; Delaney *et al*., 2015).

Pyrosequencing process can be divided into three steps: (i) PCR amplification and tagging using a biotinylated primer, (ii) isolation of the PCR product with streptavidin beads and hybridization with a sequencing primer, and (iii) sequencing. During the sequencing step, nucleotides are added in a predefined order depending on the sequence of interest. The technology is based on a release of pyrophosphate (PPi) during nucleotide incorporation when complementary to the template DNA strand (the purified PCR product). An ATP sulfurylase then uses PPi and adenosine phosphosulfate to produce ATP. ATP is utilized by luciferase which converts luciferin to oxyluciferin. The intensity of produced light is detected and translated as a peak on a pyrogram (Tost and Gut, 2007**b**). Methylation percentage is then calculated from the ratio of heights of a cytosine peak (methylated signal) and the sum of cytosine and thymine peaks (methylated and unmethylated signal) for each cytosine in a CpG dinucleotide.

Methodology for determining DNA methylation analysis by pyrosequencing

Pyrosequencing is a polymerase-based quantitative real-time sequencing method used to analyze multiple sequence variations in a region of interest. In contrast to conventional Sanger sequencing that uses a mixture of the four fluorescently labeled chain-terminating ddNTPs and strand elongating dNTPs, only one nucleotide is dispensed at a time by an inkjet-type cartridge in pyrosequencing reactions using either a user defined sequence-specific dispensation order or a repetitive cyclic dispensation order of the four nucleotides for unknown sequences.

The experimental procedure of the pyrosequencing assay is simple and relatively robust and results are highly reproducible. Therefore, pyrosequencing has become a widely used analysis platform for various biological and/or diagnostic applications such as routine (multiplex) genotyping of single-nucleotide polymorphisms (SNPs),

methylation analysis of bisulfite-treated samples, bacterial typing, mutation detection, and allele quantification (Marsh, 2007).

Nucleotides added into the pyrosequencing reaction (here exemplified by a thymine) are incorporated by the DNA polymerase extending the pyrosequencing primer when they are complementary to the DNA template sequence. This incorporation releases PPi, which is used together with APS by an ATP sulfurylase to produce ATP. ATP will be subsequently used by luciferase to oxidate luciferin to oxyluciferin generating a proportional light signal. Unincorporated nucleotides are degraded by apyrase to avoid unspecific background signals. A key step in the development of applications for pyrosequencing was the addition of a single-stranded DNA binding protein to the reaction mixture (now also included in the commercial kits), which led to a substantial increase in read length and overall greater accuracy through the reduction of the formation of secondary structures and mispriming (Dupont *et al*., 2004). Pyrosequencing has been demonstrated to be very reproducible if assays are performed in a quality-controlled and standardized fashion including enough input DNA for methylation analysis (Dupont *et al*., 2004). Pyrosequencing can also be used for screening of differential DNA methylation between two sample groups by creating pools stratified for clinical parameters of interest, for example, cancerous versus matched peritumoral tissue (Dejeux *et al*., 2007).

A (pyro) sequencing primer is subsequently annealed to this template, and the sequence is synthesized one nucleotide at a time. The light signals are then generated by the enzymatic cascade by extending the 30 ends of the nascent strand described above. It should be noted that the nucleotide dATP acts as a natural substrate for luciferase (although less efficient compared to ATP). Therefore, the a-SdATP analogue is used as nucleotide for primer extension as it is equally well incorporated by the polymerase.

Pyrosequencing can analyze almost any polymorphism in the amplified sequence. As the expected sequence is in most cases known a priori, the sequence to analyze is simply entered into the software creating automatically a dispensation order, and once the sequencing reaches this polymorphism, both nucleotides of the variable position will be added successively and their proportional luminometric signal quantified by the software.

Since all the enzymatic reactions are quantitative, the intensity of the bioluminometric response is directly proportional to the amount of incorporated nucleotides: the incorporation of two identical consecutive nucleotides will have double intensity (and therefore peak height in the resulting pyrogram) compared to the signal of single nucleotide incorporation. This quantitative nature of the results is the most important characteristic of the pyrosequencing technology because it allows performing quantitative applications such as DNA methylation analysis. Furthermore, as pyrosequencing proceeds at a rate of one dispensation per minute, results on the presence and abundance of variable nucleotides will be available between 10 and 60 minutes after launching a pyrosequencing reaction. The total time to results starting from the PCR amplification is commonly below 3–4 hours and therefore much faster than conventional Sanger sequencing.

Conclusion

Several methods for determining DNA methylation have been developed over the past two decades. However, techniques that will gain the most popularity are those that are commercially available with an easy-to-use tools or that are not too technically demanding and that require equipment that is readily available at most research institutes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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