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DNA Methylation Screening and Analysis

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Abstract

DNA methylation is an epigenetic form of gene regulation that is universally important throughout the life course, especially during in utero and postnatal development. DNA methylation aids in cell cycle regulation and cellular differentiation processes. Previous studies have demonstrated that DNA methylation profiles may be altered by diet and the environment, and that these profiles are especially vulnerable during development. Thus, it is important to understand the role of DNA methylation in developmental governance and subsequent disease progression. A variety of molecular methods exist to assay for global, gene-specific, and epigenome-wide methylation. Here we describe these methods and discuss their relative strengths and limitations.

Keywords

DNA methylation; Epigenetics; Gene regulation; Bisulfite sequencing; Epigenomics; Gene–environment interaction

1. Introduction

Epigenetic studies provide evidence for the role of environment and nutrition in developmental processes. While DNA sequence is more or less permanent, via epigenetic mechanisms gene expression is subject to differential regulation that is influenced by uterine environment, maternal diet, and chemical exposures (1, 2). Epigenetic modifications include chromatin folding and attachment to the nuclear matrix, packaging of DNA around nucleosomes, covalent modifications of histone tails, and DNA methylation. The influence of noncoding RNAs and regulatory small RNAs on gene transcription is also increasingly recognized as a key mechanism of epigenetic gene regulation (3). A single or a combination of epigenetic modifications may influence developmental gene regulation at any given time.

DNA methylation is an epigenetic modification of high interest, as it undergoes several changes during development that direct differentiation and organogenesis. It is particularly important to note, therefore, that DNA methylation is both tissue- and species-specific. DNA methylation occurs when DNA methyltransferases covalently attach a methyl group to the carbon-5 position of cytosine bases, producing 5-methylcytosine. Not all cytosine residues are methylated, however, and in mammals methylation is most common at CpG sites (4). CpG islands are rich in CpG sites and are often located in the promoter region of genes. This has the ability to influence gene expression, often through the silencing of transcription. Recent studies find evidence for partial methylation of nonCpG cytosines especially in pluripotent embryonic stem cells, indicating the role of novel epigenetic mechanisms during differentiation and development (5). Thus, identifying DNA methylation patterns for both CpG and nonCpG sites during gestation is important for understanding developmental regulation.

Epigenetic marks, including CpG methylation are generally stable in somatic cells; however, during at least two developmental time periods, the epigenome undergoes extensive reprogramming. These critical windows of development include gametogenesis (6) as well as early pre-implantation (7). At gametogenesis genome-wide demethylation occurs during the development of the primordial germ cells (6). In the mouse this process occurs from E11.5 to E12.5 (reviewed in ref. 8). In the murine male germ cells, de novo methylation occurs around E16-18.5, whereas, in female germ cells de novo methylation occurs after birth in mature oocytes. This demethylation and remethylation cycle is thought to erase previous paternal imprints and reestablish sex-specific imprints. It may also be important for clearance of acquired epigenetic marks via genetic factors, environmental exposures, or disease state.

At fertilization both parental genomes undergo further epigenetic modifications. First, the paternal genome, which prior to fertilization exists as a single copy and is densely packaged, exchanges protamines for histones (7) and undergoes active demethylation prior to DNA replication (9, 10). Second, the maternal genome, which prior to fertilization exists in two copies and is arrested at metaphase II, completes meiosis and undergoes passive demethylation after several cleavages (8). This wave of epigenetic demethylation is thought to restore totipotency of the fertilized egg; however, some sequences are protected from epigenetic reprogramming at fertilization, including imprinted genes, repeat sequences such as IAPs, and heterochromatin near chromosome centromeres (7, 9, 11, 12). De novo methylation of both parental genomes occurs around implantation, with the embryonic lineages, such as the inner cell mass, showing hypermethylation in comparison to extra-embryonic lineages, such as the trophectoderm.

As a result of these waves of epigenetic reprogramming, time-sensitivity is important when studying DNA methylation during development. First, when collecting embryonic tissues during pregnancy, gestational date is of high concern. It is important to collect DNA samples on a date that is appropriate for the study hypothesis. For example, collection of somatic DNA prior to the reprogramming stage at fertilization allows for studies of pluripotency during development, but will not yield reliable information about methylation during development because the DNA is highly demethylated. Collection of DNA during in vivo studies, however, will likely occur after the reprogramming has at least begun to occur, when the embryo is large enough to yield higher amounts of tissue and extraction is simpler.

Second, it has been demonstrated that DNA methylation during embryonic reprogramming is environmentally sensitive (reviewed in refs. 12, 13). If methylation patterns at the time of organogenesis are of concern, then the organisms must be exposed prior to the reprogramming phase in order to test their gestational effects on DNA methylation. Finally, since the F2 germline is directly affected by exposure of the grandparent generation, transgenerational studies must be carried out to the F3 generation (14).

This chapter serves as an introduction to methods used in developmental toxicology to screen for DNA methylation. Subheading 2 provides an introduction to global methylation, and includes a detailed procedure. Subheadings 3 and 4 include information about methodologies in gene-specific and epigenome-wide screening, respectively. These sections do not follow the traditional methods format used throughout this volume. This is because these methods are often proprietary and/or kit based, involving newer technologies that are constantly evolving. However, a general description of these methods and platforms is provided along with advantages and disadvantages of their use. Overall, this chapter allows readers to select an appropriate method for DNA methylation screening and provides the necessary resources to design methylation experiments for developmental studies.

2. Analysis of Global Methylation

The Luminometric Methylation Assay (LUMA) is a tool to measure absolute levels of DNA methylation in a given genome. It provides a quantitative measurement of global methylation with only 250–500 ng of DNA input, and can be performed on any species without a reference genomic sequence (15). LUMA uses differential restriction digests based upon methylation sensitivity to detect the overall methylation percentage. Two restriction enzymes, *HpaII* and *MspI*, cleave the sequence 5'-CCGG-3' (16). While *MspI* is methylation insensitive and will cleave at both methylated and unmethylated sites, *HpaII* is methylation sensitive and will only cleave unmethylated sites (17). The ratio of these two values provides the global methylation values and is measured via the incorporation of nucleotides into restriction sites using the Pyrosequencing™ platform.

LUMA has several strengths that influence its widespread usage. One of the strengths of LUMA is its internal standard, using *EcoRI* digestion of the DNA (18). This allows researchers to have slightly variable amounts of DNA yet still ensure equilibration of digests because they are calculated as ratios relative to *EcoRI* digestion. Because *EcoRI* is not methylation sensitive, it should cleave similarly amongst all samples. Additionally, LUMA is high throughput—up to 48 samples can be run on the Pyrosequencing™ platform in under 20 min. Finally, as LUMA is a global rather than a gene-specific assay, it can be performed on species without a reference genome (15).

The use of LUMA, however, is not without its drawbacks. For one, the assay only detects methylation differences within CCGG sites. Several groups have cited this as a potential source of bias, as these sites are not distributed uniformly throughout the genome nor do they exhaust all of the CpG sites in the genome (16, 18, 19). However, the sensitivity of the assay is high enough to detect minute variation between species and individuals and thus still remains highly acceptable in the literature (20). LUMA results have also been validated with other global methylation measures and have yielded correlated results (19, 21–23). Additionally, the LUMA assay can be labor intensive. Previously, the restriction-digested DNA used to be subjected to Southern blotting and polymerase chain reaction (PCR) for analysis on all of the fragmented DNA (24). However, with technological advances the Pyrosequencer™ has made the process simple and quick for those who have access.

2.1. Materials

2.1.1. Isolation of Genomic DNA: Phenol/Chloroform Extraction

- Tissue samples (embryo, visceral yolk sac (VYS), or pooled microdissection).
- Sonicator or pestle to lyse tissue in tubes.
- Heat block or water bath.
- 1.5-mL Eppendorf tubes.
- 2-mL Phase Lock Gel Tubes.
- Buffer ATL (Qiagen).
- Proteinase K.
- RNase A, 100 mg/mL.
- Chloroform.
- Phenol/chloroform/isoamyl alcohol (PCI), 25:24:1.
- Centrifuge.

- 100% EtOH.
- 70% EtOH.
- Sodium acetate buffer, 3 M.
- TE buffer (Tris–EDTA), pH 8.0.
- Spectrophotometer that can detect [nucleic acid], such as NanoDrop (Thermo Scientific).

2.1.2. Restriction Digest

- Lowly methylated DNA controls (EpigenDx).
- Highly methylated DNA control (EpigenDx).
- *EcoRI* (20 U/μL).
- *MspI* (20 U/μL).
- *HpaII* (10 U/μL).
- 10× Buffer Tango™ with BSA (Fermentes).
- Nuclease-free water.
- Isolated genomic DNA samples.
- Incubator, heat block, water bath, or thermocycler.
- 0.5-mL Eppendorf tubes, or PCR plates.

2.1.3. Pyrosequencing for LUMA Assay

- Annealing buffer (Qiagen).
- Pyro plate.
- Nucleotides (A, C, G, T).
- Pyrosequencing enzyme reagent (Qiagen).
- Pyrosequencing substrate reagent (Qiagen).
- Capillary tips.
- PyroMark™ Q96MD software (Qiagen).
- Pyrosequencing™ Q96 platform (Qiagen).

2.2. Methods

2.2.1. Isolation of Genomic DNA: Phenol/Chloroform Extraction

1. Tissue samples should either be processed fresh or flash frozen and stored without solution in Eppendorf tubes at –80°C. When working with whole embryos or VYS, one sample per vial should suffice (will yield up to 1,200 ng of DNA for embryos and up to 500 ng of DNA for yolk sacs). However, microdissections must be pooled.
2. Remove the tissue from the freezer and allow time to thaw (if necessary).
3. Set the heat block or water bath to 50°C to allow time for it to warm up to temperature.
4. Add 540 μL Buffer ATL to each sample tube.

5. Lyse or sonicate the tissue and Buffer ATL.
6. Add 60 μ L Proteinase K and vortex.
7. Put sample tubes into the 50°C heat block or water bath and allow to incubate overnight.
8. The next day, remove the tubes from the incubation and allow to cool to room temperature.
9. Set the heat block or water bath to 60°C to allow time to warm to temperature.
10. Add 12 μ L RNase A to each sample tube and allow to sit for 10 min at room temperature.
11. Centrifuge 3 phase-lock gel tubes for each sample so that the contents settle and label accordingly. Also label a 1.5-mL Eppendorf tube for each sample.
12. Transfer the samples to the first set of labeled phase-lock gel tubes and add 600 μ L of PCI. (PCI should be handled in a fume hood).
13. Shake the samples vigorously for 15–20 s and centrifuge on high for 2 min.
14. Remove the tubes from the centrifuge. The top and bottom phase should be readily separated. Transfer the top phase of each sample to a new, labeled phase-lock gel tube.
15. Again, add 600 μ L of PCI, shake vigorously for 15–20 s, and centrifuge on high for 2 min.
16. Again, transfer the top phase to a new, labeled phase-lock gel tube.
17. Add 600 μ L chloroform, shake vigorously for 15–20 s and centrifuge on high for 2 min.
18. Transfer the top phase to the labeled 1.5-mL Eppendorf tubes. Approximately 500 μ L of solution should be remaining in each tube at this time.
19. Add 50 μ L of the 3 M Sodium Acetate Buffer to each tube and vortex.
20. Add 1 mL of 100% ethanol to each tube and invert several times to mix thoroughly. Centrifuge on high for 2 min.
21. Carefully decant, vacuum, or pipette the supernatant away into a waste container to leave only the pellet in the bottom of the tube.
22. Add 1 mL of 70% ethanol to each tube, invert several times to mix, and centrifuge on high for 2 min. Carefully remove the supernatant to a waste container, leaving the pellet in the bottom of the tube. Repeat this again and allow the pellet to dry for 10 min by leaving the tube open on the bench.
23. Add 50 μ L TE buffer and place into the heat block or water bath to incubate for 1–2 h. This ensures full suspension in the solution.
24. Use a spectrophotometer that can detect nucleic acid concentration, such as a NanoDrop, to calculate the DNA concentration in each sample. Try to use as little sample as possible in this process.
25. Store samples at 4°C until use.

2.2.2. Restriction Digest

1. If you will be using an incubator, water bath, or heat block to incubate your samples, set it to 37°C so that it has time to warm to temperature.
2. Retrieve *EcoRI*, *HspII*, *MspI*, Tango buffer, and highly and lowly methylated DNA samples from the freezer to give them time to thaw.
3. Create two master mixes and vortex (see Note 1):
 - a. Mix A: For each sample include 2 µL of 10× Tango Buffer™, 0.25 µL *EcoRI* (20 U/µL), and 0.5 µL *HpaII* (10 U/µL).
 - b. Mix B: For each sample include 2 µL of 10× Tango Buffer™, 0.25 µL *EcoRI* (20 U/µL), and 0.25 µL *MspI* (10 U/µL).
4. Label two tubes for each sample, one as “A” and one as “B”. Make sure to prepare enough solution for Mix A and Mix B to include lowly and highly methylated DNA controls.
5. Pipette 2.75 µL of Mix A into the corresponding sample tubes and pipette 2.5 µL of Mix B into the corresponding sample tubes.
6. Calculate the amount of sample needed to yield 300 ng of DNA, and pipette that amount into each tube for Mixes A and B.
7. Bring the total volume of each tube to 20 µL by adding nuclease- free water. Note that the amount of water added to the tubes will differ for Mix A and Mix B due to differing volumes of restriction enzymes.
8. Allow the samples to incubate at 37°C for 4 h. If completing the sequencing step after the 4 h incubation is not possible, store the samples at 4°C overnight.

2.2.3. Pyrosequencing for LUMA Assay

1. Add 15 µL of Annealing Buffer to each sample well or vial.
2. Take 8 µL of the Annealing Buffer/restriction digests and pipette these into a Pyro plate in duplicate (see Note 2). Samples should be run in duplicate to account for inter-well variances. You should have four wells for each sample and lowly or highly methylated control: two from Mix A and two from Mix B. Only apply Annealing Buffer into the well in the upper right corner of the plate, to serve as a control for nucleotide degradation.
3. Open the PyroMark™ Q96MD software and set up a new assay, to be named the LUMA assay. Establish the nucleotide dispensation order as the following: GTGTCACATGTGTG. This sequence eliminates background with the first nucleotides, provides a standard for calibration of calculations using C and A to complement *EcoRI* restriction sites, and assesses differential methylation using nucleotides 9 and 10 by complementing the *MspI* and *HpaII* restriction sites.
4. Set up a new SNP run. Highlight and activate the wells that will be used in the on-screen template to correspond with your Pyro plate. Make sure to select the LUMA assay for all activated wells.

¹Addition of restriction enzyme may differ based on the concentration of enzymes. The reaction for methylation sensitive and insensitive cleavage requires a total of 5 units (U) of *HpaII*, 5 U of *MspI*, and 5 U of *EcoRI*.

²When performing experiments looking at differences in global methylation percentage between control and treated groups, it is prudent to include both on each and every plate that is run through the Pyrosequencer™. Treatment may greatly affect the methylation percentage, and thus confirmation is important. If both controls and treated samples are included, it is easier to detect whether methylation differences between groups are attributed to error or to treatment.

5. Using the software to calculate the amount of each reagent needed, pipette the required amount of nucleotides and pyrosequencing reagents into the capillary tips and put the tips into the cartridge. Insert the cartridge into the Pyrosequencer.
6. Insert a test plate into the Pyrosequencer and set the software to run a test dispensation. Make sure that the test dispensation shows droplets on all six test wells, and that the droplets are located within the perimeter of the wells.
7. Remove the test plate and insert your samples plate. Press the Run button to activate your run. The run should take approximately 16 min.
8. After the run has finished, click into each well individually. A unique pyrogram will have been produced for each well, containing a series of peaks that corresponds with nucleotide integration and thus methylation (see Fig. 1). The well containing only the Annealing Buffer should have a fairly flat and constant pyrogram.
9. Look at the “Data Analysis Mode” box on the screen and choose to analyze in AQ mode. Make sure to select all sample wells and click “Analyze”.
10. In the upper right-hand corner of the screen is a tab titled “peak heights”. Select this tab and export this data.

2.2.4. Analysis of Data

1. Open the file with the exported peak heights. Microsoft Excel is just one of the programs that will be able to do this, but any spreadsheet software would be appropriate. Make sure to open the file as delimited and separated by semicolons so that the different values will appear in the appropriate rows and columns of the spreadsheet.
2. Focus solely on columns 9 and 10 of the spreadsheet, as these correspond with the nucleotides that will differ depending on methylation status. For each row, calculate the fraction produced by nucleotide 10 (G) divided by nucleotide 9 (T). Once the ratio is calculated for each duplicate, calculate the average % methylation.
3. To determine the global methylation percentage for each sample, use the following formula: $1 - [(HpaII(G)/EcoRI(T))/(MspI(G)/EcoRI(T))] \times 100$, or simply $[1 - (Mix A/Mix B)] \times 100$ (see Notes 3 and ⁴).

3. Gene-Specific Methylation Analysis

Gene-specific investigation of DNA methylation can offer important information as to the underlying processes that may normally help to determine cell fate and function, or if altered, may provide unique insight as to the affected biological processes. Many studies have begun to locate methylation-sensitive (or labile) genes, often identified by changes in mRNA expression at various time points of development or by recognizing CpG islands in their promoter regions. First, cancer research has aided in the identification of such genes, as many of the regulatory pathways in cancers and embryonic development are shared. Wnt,

³On occasion, negative global methylation percentage values may be obtained. Most often, these negative values are the output of the lowly methylated controls. For samples that have very low methylation percentages (<10%), it is possible that the differences in restriction digests are so minute that the peak height value may be almost indistinguishable. In these cases, a negative methylation value is adequate as a control. However, if samples yield negative values, this is either in error or it may suggest that the methylation percentage of the tissue at that stage in development is too low for reliable detection.

⁴To determine validity, the highly methylated control should have a global methylation percentage above 80% and the lowly methylated control should have a value below 15%. Any deviation from these values may indicate a large amount of error in the digestion or the Pyrosequencing™ steps. The expected percent methylation for each sample varies by tissue, but whole embryo values tend to be significantly higher than yolk sac values.

HOX, and various other pathways crucial to both cancer and development continue to be thoroughly examined for methylation-labile genes (25). Second, several key studies have demonstrated an interaction between environment exposure and gene-specific methylation changes (as reviewed in ref. 26), including the role of dietary change and modified expression of imprinted genes (27).

It is important to recall that gene-specific methylation may be tissue-specific, and one cell type does not fit all. Tissue-specific variation of gene regulation controls differentiation, and DNA methylation may differ between all of these genes in all of these tissues. Candidate gene investigation of methylation is appropriate for researchers looking at a relatively small number of genes. However, as the number of genes of interest increases along with the number of tissues of relevance, gene-specific investigation of methylation can be very costly and time consuming. With increasing simplicity and decreased costs of modern technology, epigenome- wide approaches to examination of DNA methylation may be more appropriate for large-scale screening, especially when knowledge about the relevant subject may be in its infancy. However, gene-specific assays still provide a relatively quick and cost-effective method for epigenetic investigation.

3.1. Bisulfite Conversion

One of the most common methods for determining methylation status on DNA sequences is sodium bisulfite conversion (28, 29). Due to similarities in base pairing characteristics between methylated and unmethylated cytosines on CpG dinucleotides, standard methods cannot distinguish between different methylation states. The addition of sodium bisulfite to DNA fragments aids in the deamination of unmethylated cytosine residues to uracil. Amplification via polymerase chain reaction then incorporates thymine (30, 31). Methylated cytosines, however, remain unconverted during the treatment, leading to differential sequences dependent on methylation status.

It is important to note that quantitative methylation analysis is contingent upon complete bisulfite conversion. Without complete conversion, unmethylated cytosines can be mistaken for methylated residues and result in biased methylation profiles. To achieve proper conversion, original procedures subjected DNA to high bisulfite salt concentrations, high temperatures, and low pH settings. These harsh conditions often required high DNA input to offset the high degree of DNA fragmentation and loss. Therefore several commercially available kits are now available, which provides fast, efficient conversion with relatively low DNA input and minimum DNA loss. The most common kits include the EpiTect® Bisulfite Kit from Qiagen and the EZ DNA Methylation™ Kit from Zymo.

3.2. Cloning and Sequencing

Cloning of regions of interest followed by Sanger sequencing is considered the “gold-standard” for gene-specific methylation analysis (32). This method of DNA methylation analysis has been described previously in the *Methods in Molecular Biology* volume (33). Many studies have utilized this technique for the analysis of relevant genes during development (34, 35). This procedure requires the use of bisulfite converted DNA, and the region of interest is amplified and inserted into sensitive vectors. Manufacturers of cloning vectors utilize staining as an indicator of amplicon insertion, making the controls of the process easier. The products are then sequenced to determine allele-specific methylation patterns and percentage of methylated cells in the region of interest.

Cloning has several strengths and limitations. One universal criticism is that many clones are required to result in a quantitative methylation value. There is a great deal of variability in the number of clones used in the literature, but a minimum number of clones ($N = 10\text{--}20$)

is required to reliably detect interindividual variability in methylation. As new technology pertaining to methylation analysis has been released, the amount of time required for each analysis has been greatly reduced. Cloning is much more time-intensive than the other methods currently available on the market. Lastly, clone sequencing data may produce several sources of error. Controls are required to reduce bias from multiple copies of the same cloned sequence, slips in sequence reads due to homopolymer tracks or ambiguity, and incomplete conversion of the sequence (36). However, there are programs available to increase the reliability of the data by accounting for these issues, such as BiQ Analyzer by the Max-Planck-Institut Informatik, that have been utilized for verification of developmental data (34, 36). With the use of such controls, data reliability greatly increases. Because cloning is limited only by the relatively longer read length of the Sanger sequencer, it is the method of choice for longer regions of interest and for regions with very high density (37).

3.3. Pyrosequencing™

Pyrosequencing™ is a high-throughput quantitative method used for bisulfite sequencing. It is a method widely used in cancer research and has also been employed in several developmental studies (38, 39). Similar to cloning, pyrosequencing requires the use of bisulfite converted DNA. Using PCR, the DNA is amplified and tagged using a primer that is biotinylated. This PCR product is mixed with streptavidin beads, which form complexes due to biotin's high affinity for streptavidin binding. These DNA-bound beads are purified and isolated using a Vacuum Prep Tool™ by Qiagen and then dispensed into pyrosequencing plates that contain sequencing primer. The plate is inserted into the Pyrosequencer™, and nucleotides are added in the order of the sequence of interest. In addition to nucleotides, beads are incubated with enzymes such as DNA polymerase, ATP sulfurylase, luciferase, and apyrase, and with substrates such as adenosine 5' phosphosulfate (APS) and luciferin. Pyrosequencing technology is based on the release of pyrophosphate (PPi) when nucleotides incorporate into the sequencing primer only if it is complementary to the template DNA sequence. Unincorporated nucleotides are degraded by apyrase before the next nucleotide dispensation occurs. In the presence of adenosine phosphosulfate (APS), ATP sulfurylase utilizes PPi to produce ATP. In turn ATP drives the conversion of luciferin to oxyluciferin by luciferase (40). The intensity of light produced by this reaction and detected by the Pyrosequencer is contingent upon the amount of nucleotide incorporation at specified sequences surrounding CpG sites, and translated as a peak on the Pyrogram. From this information, methylation percentage can be calculated by the platform.

One source of error when pyrosequencing is variation in the number of reads obtained for each sample, often influenced by DNA quality and/or secondary structure (41). Another shortcoming of pyrosequencing is a lack of resolution in homopolymer regions, as identical nucleotide incorporation in a sequence can be blurred across various nucleotide steps (41). Lastly, pyrosequencing is incredibly sensitive and can often result in failed signals due to errors or perceived failed bisulfite conversions. This can be due to various mechanical errors such as being bumped during a run or, more commonly, due to low template availability. Thus, high-quality primer design and proper template amplification is crucial for each assay.

Conversely, pyrosequencing has many positive features. For facilities that complete many genetic analyses, pyrosequencing is useful for DNA methylation as well as single nucleotide polymorphism (SNP) analyses. It is far less time consuming than cloning, taking hours instead of days. Pyrosequencing is also sensitive enough to provide accurate reads with each run, unlike cloning which depends on the number of reads carried out (42). All reactions use the same reagents, and only primers vary between different assays. These reagents are fairly expensive, but can be used for many assays. Primer design is fairly simple for pyrosequencing, as many companies have created software to assist in assay design. These software, including the PyroMark Assay Design by Qiagen, offer many checks in order to

reduce the amount of complications such as dimerization during runs. Likewise, companies have now created verified primers for purchase for research use.

3.4. Mass Spectrometry

Mass spectrometry methylation assays provide a sensitive method of detection based on difference in fragment weights that have been cleaved based upon methylation status. The Sequenom MassArray platform with EpiTYPER[®] analysis software is one such assay. It requires the use of bisulfite converted DNA, and primers designed in regions without CpG nucleotides. A T7 promoter site is added to all forward primers and the target is amplified using PCR. These products undergo transcription by T7 RNA and DNA polymerase with simultaneous cleavage by RNase A, and the additional dNTPs are removed using shrimp alkaline phosphatase. Deoxycytidine triphosphate (dCTP) or deoxythymine triphosphate (dTTP) is incorporated into the RNA transcript, and RNase A will only be able to cleave at sites immediately 3' of the incorporated dCTP or dTTP residues. These fragmented transcripts are run through mass spectrometry for analysis. The methylated and unmethylated fragments will differ in mass due to this differential cleavage, and the analytical software quantifies methylation percentage.

The Sequenom MassArray platform with EpiTYPER[®] has been used in developmental studies (43–45). It is highly sensitive and has the ability to sequence reads up to 600 bp, which is considerably longer than other methods (46, 47). Studies have shown variable site specificity of the assay, with studies demonstrating ranging values from 75 to 95% concordance when validated with other sequencing methods (45, 48).

3.5. qPCR Arrays

Quantitative PCR (qPCR) provides another means of methylation quantification. qPCR operates using fluorophore-labeled probes that emit fluorescence when bound to a complementary DNA sequence. One assay that utilizes qPCR to determine localized methylation is Qiagen's EpiTect[®] MethyLight Assay, which is a system that uses probes specific to either methylated or unmethylated sequences. DNA is bisulfite converted, and the TaqMan[®] probes are designed as complementary to either the methylated and converted or unmethylated and converted sequence (49, 50). These probes are labeled with a different fluorophore in order to distinguish differential binding. Quenchers are added to the probes to hide fluorescence and are subsequently removed during hybridization to the DNA. Thus, if the localized sequence exhibits high methylation, it will bind the complementary probe and emit the fluorophore in a quantitative fashion.

The MethyLight Assay has several strengths as well as limitations. Many cancer studies have utilized MethyLight to determine CpG methylation in repetitive regions and in genes that are also developmentally relevant (51–53). However, very few published developmental studies have used MethyLight at this time. MethyLight provides a validated and consistent assay for methylation that is widely used and published. Studies have concluded that MethyLight exhibits high sensitivity, and that results are consistent with other methylation assays (50).

One complication in the use of MethyLight depends on the number of CpG sites in the amplicon. For a region that has many CpG sites, MethyLight cannot provide exact quantitation of methylation percentage without creating a greater number of probes specific to each possible methylation pattern (50). For example, if the region of interest contains $X = 3$ CpG sites, then it would be necessary to create eight (2^X) specific probes to assay the exact placement of methylation within your region. This can become potentially costly for sequences with a larger number of CpG sites. However, if it is only desired to determine

whether a region is highly or lowly methylated, general primers may be able to hybridize to the sequence. Likewise, if the region of interest contains only a few CpG sites, or if you plan to assay this region many times with many samples, MethyLight provides a fairly simple and relatively inexpensive way to conclude a high-power study.

4. Epigenome-Wide Arrays and Analysis

The systematic screening of important developmentally labile loci for differential gene expression is critical in understanding both normal and abnormal development. Abnormal growth, especially in the developing fetus, may affect organ function and induce metabolic changes that predispose the fetus to disease later in life. The fetal basis of adult disease theory proposes that a divergence between the prenatal environment and postnatal environment increases the risk of adult diseases such as cancer and diabetes (54). Epigenetic regulation, including DNA methylation, histone modification, and small RNA interference, is an important mechanism further supporting the role of intrauterine environment on developmental plasticity (26). Alternatively known as the developmental origins of health and disease (DOHaD), this field originally emerged from large epidemiological studies on infant and adult mortality rates (55). As the field develops to incorporate in-depth biological evidence, there will be a need to work with various animal models to avoid unethical use of sensitive human fetal samples. There will also be a need to understand and compare differential gene expression and epigenetic marks among varying tissues and animal models. Therefore, high-throughput assays and technologies are crucial in advancing the field of developmental biology.

In the past decade, several novel approaches have been introduced for epigenetic and epigenomic analysis that aid in understanding development and fetal-based diseases. They apply basic techniques that are commonly used, such as bisulfite conversion, digestion with methylation-sensitive enzymes, and anti- 5' methylcytosine antibodies, and combine them with tiling arrays and deep sequencing (56, 57). Many of these methods have increased accuracy, sensitivity, high-throughput capacity and overall cost-efficiency for quantitative analysis of large genomic regions or candidate genes. Innovative epigenome-wide platforms and arrays utilize either biased or unbiased approaches for analysis. An unbiased approach reveals the full regulatory network at the level of the whole genome while biased analysis limits analysis to certain loci or regions of the genome, such as promoter regions, revealing only a partial picture of epigenomic regulation. Large-scale epigenome- wide analyses continue to be important strategies for cancer research, but can equally be applicable for uncovering changes in gene expression during early development (58–60).

4.1. General Techniques

Microarray technology provides a rapid survey of altered gene expression for a particular phenotype or exposure. In general, it is a hybridization-based assay that analyzes thousands of sequences simultaneously without requiring a large sample volume. Fluorescently labeled nucleic acids are hybridized to reporter molecules such as oligonucleotides, which are built onto a solid surface (56). After an initial wash to help reduce nonspecific signals, the microarrays are scanned under a confocal fluorescent microscope at wavelengths appropriate for the given fluorescent labels. The subsequent fluorescent image is measured for intensity at every spot, resulting in raw data comparing the extent of hybridization of experimental samples to a reference sample (61). Microarray data can be interpreted only after raw results undergo background correction and data normalization for comparable, accurate results. Several up-to-date software packages are available for interpretation and visualization of epigenomic data including Gene Set Enrichment Analysis (57).

Next generation sequencing is an emerging technology based off of the Human Genome Project that took place throughout the 1990s. Unlike the conventional Sanger sequencing, next generation or deep sequencing technologies rapidly produce large amounts of sequence data at relatively low costs. Biases created by specific probes, allele-specific differences, and amplification that appear in microarray technology are bypassed with sequencing technology (57). Given that the methylation status is analyzed at every cytosine, deep sequencing provides great resolution for methylation profiles. However, the high cost of total sequencing runs and heavy reliance on computational analysis limit the use of genome-wide sequencing (30).

4.2. Affinity- and Restriction Enzyme-Based Arrays

Tiling arrays use photolithographic technology for unbiased (whole genome) to biased (promoter or custom) genome coverage. With this technology, short probes span the genome with high specificity and high resolution. However, short probes may be subject to increased random signals compared to longer oligonucleotides (30, 56, 62). These technologies often use a minimum number of arrays for human whole-genome profiles, with standard arrays that are relatively affordable (63). Some commonly used chip techniques used for epigenetic studies include chromatin immunoprecipitation (ChIP), methylated DNA immunoprecipitation platforms, as well as methyl-binding protein immunoprecipitation platforms. These platforms are commercially available by Affymetrix.

Immunoprecipitation platforms provide unbiased epigenome-wide analysis of histone modifications. Once histones are cross-linked to DNA using formaldehyde and sonicated, specific antibodies are used to immunoprecipitate histone modifications for analysis of interest. After antibody pull down, DNA-protein crosslinks are reversed, and DNA fragments are purified (64). For hybridization to a platform chip, fragments must be amplified either by whole-genome amplification (WGA), T7 polymerase-based amplification, or by linker-mediated PCR (65). In a study using a custom ChIP array targeting highly conserved noncoding elements (HCNEs), mouse embryonic stem cells exhibited high levels of trimethylation at H3K27 and H3K4 loci, also known as bivalent domains, at genes critical for development and pluripotency. Although methylation at H3K27 is repressive and H3K4 is activating, presence of these bivalent domains indicate repression at developmental genes that are poised for activation after differentiation (66). Other unbiased epigenetic approaches include methylated DNA immunoprecipitation (MeDIP). Sheared or enzyme-digested DNA fragments are subject to anti- 5' - methylcytosine antibody binding for enrichment of methylated cytosine regions of the epigenome. Methylated fragments are purified and amplified for higher DNA yields (56, 67). A major limitation to immunoprecipitation techniques in epigenome-wide analysis is the quality of the antibody. Without a high-quality antibody, improper enrichment of DNA-protein will occur (64, 65). For an epigenome-wide profiling experiment, the antibody should be able to enrich significantly more than the background for the best analysis. In general, these immunoprecipitation techniques require the availability of large sample volumes and only measure relative enrichment of epigenetic markers.

NimbleGen, like Affymetrix, also utilizes photolithographic technology, but involves long 60-mer oligonucleotide probes. Agilent arrays, on the other hand, use inkjet technology with longer probes. The longer oligonucleotides reduce background noise, with the disadvantage of having reduced probe density. The ability for dual hybridization on a single chip controls for inter-array variation among samples labeled with different fluorescent dyes (30).

Restriction enzyme-based methods are often combined with other large-scale technologies for genome-wide analysis. One of the first methods used includes differential methylation hybridization (DMH), which semiquantitatively analyses CpG sites. Here, relatively little

genomic DNA can be digested by the methylation-insensitive *MseI* enzymes and then ligated with linkers. The secondary digestion of these fragments with methylation-sensitive enzymes, *BstUI* or *HpaII*, helps remove unmethylated fragments. The methylated products are first amplified by primers that anneal to the linker sequence, then fluorescently labeled, and finally hybridized to arrays (68). The *HpaII* tiny fragment Enrichment by Ligation-mediated PCR (HELP) utilizes genomic fragments digested by both *HpaII* (methylation sensitive) and *MspI* (methylation insensitive) enzymes. Both products are amplified by ligation-mediated PCR and then hybridized to microarrays (69).

4.3. Bisulfite Conversion-Based Arrays

Illumina also offers a variety of platforms for epigenome-wide analysis including the GoldenGate Methylation assay and the Infinium BeadArray. Unlike the ChIP-chip platforms, Illumina Methylation profiling is based on bisulfite converted DNA genotyping (62). Bisulfite converted DNA is measured by two probes, one that recognizes methylated cytosines and another that recognizes unmethylated cytosines. Single base pair extension allows for the incorporation of fluorescently labeled nucleotides. Adenine and thymine nucleotides are usually labeled with one dye, while guanine and cytosine nucleotides are labeled with another dye (70, 71).

The GoldenGate assay provides high sensitivity and accuracy while permitting higher sample throughput. For the GoldenGate assay, as little as 200 ng of genomic DNA can be converted and analyzed to check methylation status at 1,532 different sites in a biased approach (56). Bisulfite conversion changes unmethylated cytosines to uracil and maintains methylated cytosines as cytosines, thereby allowing for quantification of a C/T polymorphism using primers specific for methylated and unmethylated sites (57, 72). The ratio of C and T alleles can therefore be converted to a methylation percentage at a certain loci (73). For a biased approach, methylation-profiling arrays can be customized to span CpG loci near genes critical for development.

The Illumina Infinium HumanMethylation27 and Human-Methylation450 Bead Arrays provide genome-wide coverage, featuring methylation status at CpG islands, CpG shores, promoter regions, 5' UTR, 3' UTR, as well as gene bodies, spanning 14,495 and all designable Ref Seq human genes, respectively. Although semi-biased, the HumanMethylation27 Bead Chip platform can interrogate over 27,000 CpG sites at single-site resolution (56). With a low sample input of at least 500 ng, valuable samples can be used for quantitative analysis without the requirement of polymerase chain reaction. CpG methylation is calculated as the ratio between the methylated probe signal relative to the total (methylated and unmethylated) probe signal at every locus. The resulting value, known as the beta value, ranges from 0 to 1, representing unmethylated and methylated status, respectively (70). The newest methylation array, the Illumina Infinium Methylation450 BeadArray, can interrogate over 450,000 CpG sites within CpG islands, shores, and shelves, as well as nonCpG sites throughout the genome (74). At present, this technology is available for genome-wide methylation analysis in humans only (75).

4.4. Next Generation Sequence-Based Platforms

A number of high-throughput sequencing platforms are available including 454 Life Sciences pyrosequencing and Illumina (Solexa). The 454 system is a high-throughput pyrosequencing technique that produces 400,000 reads over 100 bases per read. It requires a bead-based emulsion polymerase chain reaction, which provides parallel sequencing of amplified DNA templates at every bead (63). As nucleotides are systemically incorporated during every cycle, the light intensity is recorded for each nucleotide per bead over time (76). The disadvantage to using pyrosequencing for genome-wide analysis involves the

inability to assess repetitive sequences or homopolymer tracks, as well as the potential for DNA degradation during bisulfite conversion (57). The SOLiD technology is similar to 454-system but utilizes smaller beads and can interrogate adjacent bases to help discriminate between sequencing errors and polymorphisms. However, the error rate is significantly higher than conventional methods and color-coding measurements require extensive processing (76).

The Illumina (Solexa) technology is a bridged PCR-based system that produces 40 million reads of up to 100 bases (56). Adaptor-ligated DNA templates are hybridized to primers tethered to a flow cell. A reverse complementary copy of the template can later be used for isothermal amplification in order to produce clustered amplified strands. After laser excitation of the terminator nucleotides on each cluster, fluorescent signals are detected by a charged coupled device (CCD) camera (56, 76).

Several studies have already utilized technologies such as large-scale bisulfite sequencing, ChIP sequencing, MeDIP sequencing, or MethylC sequencing for epigenomic analysis in developmental biology. When Bird and colleagues applied affinity-purified nonmethylated CpG-rich DNA to a custom array followed by bisulfite sequencing, they noticed tissue-specific methylation of CpG sites relevant to loci critical to development such as *HOX* and *PAX* genes (77). Using the Illumina Genome Analyzer, Meissner et al. (60) found that methylation profiles of mouse embryonic stem cells appeared to be well correlated to histone methylation patterns and undergo drastic changes during differentiation, especially at regulatory regions (60). Analysis of human embryonic stem cells and lung fibroblasts by MethylC-seq indicated a novel enrichment of nonCpG dinucleotides in nearly one-quarter of all methylated sites, strictly in pluripotent cells (5).

4.5. Conclusions

Epigenome-wide studies and emerging technologies continue to contribute to epigenetic knowledge of various scientific disciplines. It is well established that heterochromatic regions consisting of highly repetitive sequences are mostly hypermethylated, with methylation at 70% of CpG dinucleotides throughout the genome (78). Many of these methylated regions appear in noncoding genomic areas or may be pseudogenes. Conversely, very few promoters are methylated and surprisingly, methylation markers can appear along the length of genes and at 3' end (79). More importantly, both DNA methylation and histone-modification patterns vary by species, by tissue and with time. As a result, there is a great need for global, gene-specific, and epigenome-wide analysis in early development and during tissue differentiation and growth.

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Fig. 1. Example of a pyrogram produced during LUMA. The ratio of peaks provides methylation percentage.