Multiplex Methods to Examine Epigenetic Modifications

Introduction

The term “epigenetics” has evolved substantially. Historically, it was used to describe events that could not be explained by genetic principles. Now, epigenetics can be broadly thought of as the link between genotype and phenotype. Epigenetics changes the final product of a locus or chromosome without changing the underlying DNA sequence. Research, as of 2017, focuses on the study of covalent and noncovalent modifications of DNA and histone proteins, and how these modifications influence the structure of chromatin. DNA methylation provides a stable, heritable, and critical component of epigenetic regulation. Almost all DNA methylation in mammals occurs on cytosine residues of CpG dinucleotides. DNA methylation also partly mediates the formation of heterochromatin and plays a role in many cellular processes, including sequence silencing, the inactivation of X chromosomes, and imprinting.

Emerging evidence is connecting covalent histone modifications with longstanding epigenetic phenomena. Modifications, such as acetylation and phosphorylation, have been suggested to directly alter chromatin and cause changes in higher-order structures. Research also suggests that histone modifications recruit or stabilize the localization of specific binding partners to chromatin. Furthermore, noncovalent mechanisms provide cells with additional tools for introducing chromatic variation. These mechanisms include chromatin remodeling and incorporating specialized histone variants. The reversible nature of epigenetic modifications provides promise for therapeutic and preventative strategies, particularly for diseases previously thought to be hard-wired into the genome.

Traditional Techniques

Several tools are commonly used to investigate DNA methylation and histone modifications.

- Chromatin immunoprecipitation (ChIP) is the primary method of analyzing histone modifications and involves the immunoprecipitation of chromatin using antibodies against a transcription factor, a chromatin-associated protein, or a modified histone. Polymerase chain reaction (PCR) is then used to assess the presence, absence, or relative enrichment of a predefined sequence in the ChIP DNA.
- Microarrays (ChIP-on-chip) and sequencing the ChIP DNA support more extensive analyses that can detect epigenetic changes associated with uncharacterized transcriptional units or regulatory elements. They also generate continuous data along chromosomes, defining the extents and boundaries of genomic regions with modified histones.
- The N-ChIP technique fragments chromatin by micrococcal nuclease digestion as opposed to sonication and does not require cross-linking.
- Biotin-tag affinity purification is also used to map histone variants that are fused to a biotin ligase recognition peptide. High specificity is achieved with biotin-tag affinity purification because it relies on streptavidin pull-down, as opposed to immunoprecipitation.
- DNA adenine methyltransferase identification (DamID) is another method for mapping chromatin-associated proteins or transcription factors. In this method, the protein of interest is fused to a DNA adenine methylase, and its DNA targets are identified by restriction with adenine methylation-sensitive enzymes.
- Southern blotting was the original technique routinely used to analyze DNA methylation. However, this method requires a relatively large quantity of DNA (5-10 mg) and thus has largely been replaced by other methods.
- Bisulfite-converted DNA involves the sodium bisulfite treatment of genomic DNA, which converts unmethylated cytosine to uracil. This includes methods such as bisulfite pyrosequencing and BeadChip analysis, but the expertise, time, and cost requirements are all limitations for their use in clinical diagnosis.
• Affinity assays that precipitate methylated DNA are also used for assessing DNA methylation. These include the methylated DNA immunoprecipitation method (MeDIP), a genome-wide, high-resolution approach that utilizes anti-methyl-cytosine antibodies to immunoprecipitate DNA that contains highly-methylated CpG sites, and methyl CpG binding domain (MBD) precipitation of genomic DNA. There are also restriction enzyme methods that recognize methylated and unmethylated sequences, including the comprehensive high-throughput arrays for relative methylation (CHARM), the luminometric methylation assay (LUMA), and the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP).

**Multiplex Methods**

Combining the PCR amplification-SSOP protocol with Luminex’s xMAP® Technology results in a high-throughput, high-resolution genotyping method that can be applied clinically for the detection of different alleles. This method combines PCR and sequence-specific oligonucleotide probe (SSOP) protocols with the Luminex xMAP instruments to quantitate fluorescently-labeled oligonucleotides attached to color-coded microbeads. This proven method has been used for the detection of alleles at human leukocyte antigen loci, as well as the genotypes for multiple polymorphisms and candidate genes involved in diabetes mellitus.

While xMAP multiplexing technology is best known for profiling immune, inflammatory, and metabolic biomarkers, it can also be used for methylation analysis. xMAP Technology enables the evaluation of up to 500 analytes simultaneously in a single well, known as multiplexing. xMAP Technology uses carboxylated polystyrene microspheres that have been fluorescently dyed into up to 500 spectrally distinct sets, or “regions,” allowing them to be individually identified. In the multiplex assay environment, the microspheres act as both the surface for the solution phase assay and the spectral identifier that the instrument detects. The open architecture of xMAP Technology enables compatibility with both protein and nucleic substrates. The microspheres are individually read in a xMAP instrument. There are more than 1,400 commercially available xMAP-based kits in the xMAP Kit Finder.

Below are summaries of recent publications that explore the implications of epigenetic modifications and highlights the diverse applications of xMAP Technology, including custom multiplex assays for methylation analysis, kits used for DNA methylation analysis, and kits to quantify biomarkers.

**Custom Multiplex Assays for Methylation Analysis**

**Deep bisulfite sequencing**

A study by Kuhtz et al. used deep bisulfite sequencing (DBS) to generate methylation information on a large number of individual DNA molecules from different target genes and different samples. Most conventional techniques for methylation analysis are unable to detect small increments in epimutations in individual genes and provide the ability for only a limited number of samples and alleles to be analyzed. DBS provides the ability for massive parallel sequencing of thousands of individual DNA target molecules in a biological sample, generating methylation information for a large number of individual DNA molecules in a single experiment.

Sperm samples were collected for DBS from 54 couples undergoing infertility treatment, including 27 from men with normal sperm parameters and 27 from men with oligoasthenoteratozoospermia (OAT) syndrome. DNA was extracted from pools of 10 sperm each, and multiplex PCR was performed in 25 μL reactions for GTL2, LIT1, and PEG3 for LD bisulfite pyrosequencing. Single sperm analysis was also carried out using multiplex (BOLL, CATSPER1, OCT4, NANOG, GTL2, LIT1, PEG3, and SNRPN) PCR of single sperm DNA in 25 μL reactions. In all sperm samples, the majority of the studied genes showed normal methylation patterns with low percentages of epimutations (<1%). However, GTL2 was the best marker gene of assisted reproductive technology (ART) outcome. The average percentage of abnormally demethylated alleles was 0.2% in the normozoospermic (NC) group and 0.3% in the OAT samples that led to a pregnancy, compared to 0.5% in the NC group and 1.8% in OAT samples that did not lead to pregnancy. This resulted in a highly significant between-group difference (P < 0.0001). Therefore, epigenetic signatures can provide an additional layer of information for assessing sperm quality before infertility treatment.

**Bisulfite PCR-Luminex Method**

The bisulfite PCR-Luminex (BPL) method, is a high-throughput, high-resolution DNA methylation analysis method for the rapid analysis of DNA methylation. In 2012, Hiura et al. applied the BPL method to 21 human ovarian cancer (HOC) cell lines and 74 HOC tissues in order to determine the methylation status of differentially methylated regions (DMRs) at eight imprinted gene loci (H19, IGF2, KCNQ1, LIT1, GTL2, PEG1, PEG3 and NDN), six of which contained tumor suppressor genes (TSGs). PCR primer sets were designed for gene amplification. Oligonucleotide probe sequences were synthesized and covalently bound to carboxylated fluorescent microbeads using ethylene dichloride. Reaction outcomes were measured using the Luminex® 100/200™ instrument, and methylation assays were performed for each DMR using the conventional bisulfite treatment PCR methylation assay and combined bisulfite PCR restriction analysis (COBRA). The BPL method precisely quantified the methylation status of DMRs in this study. The average DNA methylation status of HOC and normal samples was compared for each DMR, and PEG1 from HOC samples was significantly more hypermethylated than normal ovarian tissues (normal, 30.7% ± 15.1: HOC, 45.9% ± 15.5).

To further establish the BPL method as an automated, high-throughput procedure for the detection of alterations in DNA methylation, Sato et al. compared the BPL method to the COBRA/sequencing method using the same bisulfite-treated DNA. Sperm samples were collected from a total of 337 men. Of these men, 61 had severe oligozoospermia, 67 had moderate oligozoospermia, and 209 had macroscopically-confirmed normozoospermia. Sperm DNA samples were treated with sodium bisulfite, and eight PCR primer
sets were designed for gene amplification of eight DMRs (ZDBF2, H19, GTL2, ZAC, PEG1, PEG3, LIT1, and SNRPN). Oligonucleotide probe sequences were synthesized as described above and reaction outcomes were measured using the Luminex® 100/200™ instrument. A total of 47 cases (13.9%) showed abnormal methylation at one or more imprinted loci (18 paternal, 18 maternal, and 11 cases with alterations of both maternal and paternal imprints), suggesting that methylation errors at imprinted loci are more frequent in oligozoospermic men.7

Kits for DNA Methylation Analysis

In addition to custom multiplex assays described above, kits are used for DNA methylation analysis. One example is the QuantiGene™ Plex Assay available from ThermoFisher Scientific®, which is a hybridization-based assay that uses Luminex’s xMAP magnetic beads for multiplexing of 3 to 80 RNA targets and branched DNA (bDNA) signal amplification technology. Wertheim et al. used a QuantiGene™ hybridization panel to examine the linearity and specificity of the novel microsphere HpaII tiny fragment enrichment by ligation-mediated PCR (MELP) assay. The DNA methylation pattern of 18 loci associated with the prognosis of subjects with acute myeloid leukemia (AML) was examined. Genomic DNA was digested from primary AML samples with MspI, and ligation-mediated PCR was performed. Sequential hybridization reactions for complexing amplicons onto fluorosceous microspheres and for bDNA signal amplification were performed with the QuantiGene™ 2.0 Assay. The fluorescent signals from the PCR products were analyzed on a FLEXMAP 3D™ instrument. Signals were normalized to unmethylated control loci, providing a level of methylation at a single locus. MELP accurately reflected the methylation levels at the specific loci analyzed. It also accurately segregated subjects with AML into the appropriate prognostic subgroups, indicating usefulness for simultaneous evaluation of DNA methylation of multiple loci.13

The QuantiGene™ Plex Assay has also been used to determine mRNA expression of phase II enzymes and transporters. Lu et al. examined mice lacking HNF4α (a liver-enriched key regulator of liver function) expression in order to assess a role of HNF4α in regulating the hepatic expression of phase II enzymes and transporters in mice. Total liver and kidney RNA was isolated, and mouse liver mRNAs encoding phase II enzymes (Ugts, Sults, and Gsts), and transporters were determined from the total RNA using the QuantiGene™ Plex Assay. Individual bead-based oligonucleotide probe sets, specific for each gene examined, were developed. The samples were analyzed with a xMAP-based instrument. Individual gene mRNA was then quantified using a QuantiGene™ bDNA signal amplification kit, and DNA binding of transcription factors was determined using a ProcartaPlex™ Assay. Results showed significant differences in mRNA expression of several phase II enzymes and phosphate and sulfate uptake transports, including Npt1 and Sat1.14

A similar study by Holloway et al. used QuantiGene™ Plex technology to assess the effect of Hnf4α deficiency on histone modifications.15 To determine the role of HNF4α in global and specific alterations at selected loci of representative histone modifications and DNA methylations, mouse livers lacking Hnf4α were analyzed. The enrichment of H3K4me2, H3K4me3, H3K9me2, H3K9me3, H3K27me3, and H3K4ac was determined in a set of gene loci, as these genes had been assessed in previous studies.14,15

Finally, Bando et al. developed a xMAP-based assay was used to assess the genome-wide DNA methylation status in metastatic colorectal cancer to identify possible associations between the methylation status and the clinical response to anti-EGFR treatment.16 Tumor tissue from 97 subjects with metastatic colorectal cancer was harvested, and genomic DNA was extracted. While mutations in KRAS exon 2 and BRAF V600E were analyzed by direct DNA sequencing, infrequent-RAS mutations, including KRAS and NRAS were analyzed using the Luminex assay. Genome-wide DNA methylation was then analyzed, followed by the evaluation of methylation status using previously defined markers. The Luminex assay was able to confirm the absence of KRAS codon 12, and 13 mutations in all 97 primary tumor samples.17

Kits to Quantify Biomarkers

Inflammatory Diseases

xMAP multiplexing technology is well suited for studying inflammatory diseases, particularly to assess circulating chemokines or pro-inflammatory cytokines. A study by Shaddox et al. examined the role of epigenetic regulation in individuals with localized aggressive periodontitis measured cytokine levels in lipopolysaccharide (LPS)-stimulated blood using a MILLIPLEX® multiplex assay. Significant correlations were found with DNA methylation levels of seven toll-like receptor (TLR) signaling genes, suggesting that methylation status in these specific genes may play a role in localized aggressive periodontitis.18 A separate study by Hardbower et al. examined the role of macrophages in the innate immune response to bacterial infections, using a Luminex multiplex array to assess 25 distinct chemokines and cytokines in gastric tissues from chronically infected mice deficient for ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine metabolism. Several chemokines and cytokines were significantly increased in H. pylori-infected, ODC-deficient tissues, including CCL2-5 and CXCL1, 2, and 10, suggesting that the deletion of macrophage ODC may enhance immune cell infiltration into the stomach, contributing to the significantly increased inflammation seen in bacterial diseases.19

Epigenetics has also sparked further exploration of the pathogenesis of systemic lupus erythematosus (SLE), and xMAP-based multiplex kits are often used to measure pro-inflammatory cytokines involved in the condition. Following whole-genome transcription and DNA methylation analysis in peripheral blood mononuclear cells (PBMCs), Zhu et al. analyzed serum pro-inflammatory cytokines using a Bio-Plex Pro™ Human Cytokine 27-plex assay. The 27-plex assay kit contains beads conjugated with monoclonal antibodies specific for several pro-inflammatory cytokines, including IL-17A, bFGF, IP-10, MCP-1, TNFα, and IL-8. Significant elevations were found in interferon and TLR signaling pathways and several pro-inflammatory cytokines, including TNFα and IL-6, suggesting that aberrant DNA methylation is likely relevant to the pathogenesis of SLE.20
Immune Response

Similar to inflammatory diseases, kits using xMAP Technology are also used to study the immune system, as well as diseases and pathology associated with the immune system. For example, Verschoor et al. assessed the role of circulating cytokine levels in age, acute illness, and chronic disease by comparing serum levels of several cytokines with genome-wide alterations in the DNA methylation levels of blood leukocytes from human subjects. Serum levels of several cytokines, including IFN-γ, TNF, IL-6, and IL-10 were measured from whole blood using a MILLIPLEX™ High Sensitivity Multiplex Assay. The levels of all measured cytokines were higher in the experimental group, and serum IL-10 levels showed the greatest association to DNA methylation patterns, providing evidence that circulating cytokine levels correlate with the DNA methylation patterns of human leukocytes. In another study, Jiang et al. examined the relationship among reactive oxygen species (ROS), histone transition, and seminal cytokine concentrations utilized analysis of samples with a protein chip and a Bio-Plex Pro™ Cytokine Assay. The assay validated 18 cytokines in each sample from 6,560 men. Several cytokines showed elevated concentrations in semen with high ROS levels, suggesting a potential mechanism for the increase in the ROS and seminal cytokine levels seen in defective spermatogenesis. Finally, in addition to the adult immune system, xMAP-based multiplex kits have been used in the study of the neonatal immune system, of which little is known regarding regulation of histone modifications. Bermick et al. used a BioPlex Pro™ Multiplex Immunoassay to measure protein levels of the cytokines IL-1β and TNF from cell culture supernatants, in addition to several other pro-inflammatory cytokines measured using other methods, and found elevated levels in neonates. Results suggest that neonatal immune cells exist in an epigenetic state that is distinctly different from adults, potentially contributing to neonatal-specific immune responses that leave them particularly vulnerable to infections.

Several groups have used Luminex technology kits to examine TNFα, which can be helpful for examining certain disease states. For example, Dao et al. measured maternal blood PBDE levels and cord blood TNFα promoter methylation levels to assess a potential relationship between the two. Serum samples were analyzed simultaneously for TNFα, allowing for optimal measurement of several analytes in one well. Decreased TNFα methylation in cord blood was associated with high maternal exposure to PBDEs and increased levels of TNFα protein in cord blood, providing evidence that in utero exposure to PBDEs may epigenetically reprogram immunological responses in offspring through promoter methylation. Another study by Arroyo-Jousse et al. examined type 1 diabetes using the MILLIPLEX® MAP Human Adipokine Magnetic Bead Panel 2 to quantify the levels of TNFα in blood from subjects with the disease. Subjects with type 1 diabetes showed a higher TNFα gene promoter methylation compared to healthy controls, providing evidence for the potential use of TNFα as a biomarker for early risk of the disease.

Obesity

xMAP Technology-based kits are often used in obesity studies, particularly in measuring leptin levels. One study by Pazienza et al. measured insulin, leptin and IGF-1 levels in the sera of wild-type and transgenic animals using a customized mouse MILLIPLEX® MAP assay to assess the role of isoforms of macroH2N1 in adipose tissue plasticity. Another study by Allard et al. examined the effects of maternal fasting glucose levels in the 2nd trimester on neonatal leptin levels measured cord blood leptin levels using a MILLIPLEX® Multiplex Assay. To assess whether chronic obesity induces mammary gland epigenetic reprogramming and increases mammary tumor growth, Rossi et al. measured mean tumor volume and expression of pro-inflammatory genes and DNA methylation profiles in diet-induced obesity (DIO) female mice. Serum hormones, adipokines, and cytokines, including leptin, adiponectin, insulin, IL-6, and, insulin-like growth factor 1 (IGF1) concentrations were measured using a MILLIPLEX® Assay. Finally, a study by Mosher et al. examined DNA methylation patterns along a region of the leptin gene. Plasma leptin levels were determined by radioimmunoassay and multiplex assay. Findings suggested that variations in DNA methylation along the leptin gene promoter play a role in energy signals of adipogenesis and bone metabolism.

Summary

The ability to evaluate up to 500 analytes simultaneously in a single well is critical to the study of DNA methylation, histone modifications, and genetic biomarkers, as research examining epigenetic modification often requires several targets to be simultaneously measured. Custom multiplex assays have been used in the study of DNA methylation, such as the DBS and BPL methods utilized by several groups. Kits based on xMAP Technology are used in the study of DNA methylation, specifically the QuantiGene™ Plex Assay. Finally, multiplex immunoassay kits manufactured by Bio-Rad and MILLIPORE SIGMA are commonly used to quantify genetic biomarkers, such as pro-inflammatory cytokines and leptin in the study of inflammatory diseases, immune disorders, obesity, and cancer.

References


