Bisulfite sequencing

Bisulfite sequencing (also known as bisulphite sequencing) is the use of bisulfite treatment of DNA to determine its pattern of methylation. DNA methylation was the first discovered epigenetic mark, and remains the most studied. In animals it predominantly involves the addition of a methyl group to the carbon-5 position of cytosine residues of the dinucleotide CpG, and is implicated in repression of transcriptional activity.

Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Thus, bisulfite treatment introduces specific changes in the DNA sequence that depend on the methylation status of individual cytosine residues, yielding single-nucleotide resolution information about the methylation status of a segment of DNA. Various analyses can be performed on the altered sequence to retrieve this information. The objective of this analysis is thereby reduced to differentiating between single nucleotide polymorphisms (cytosines and thymidines) resulting from bisulfite conversion (Figure 1).

Figure 1: Outline of bisulfite conversion of sample sequence of genomic DNA. Nucleotides in blue are unmethylated cytosines converted to uracils by bisulfite, while red nucleotides are 5-methylcytosines resistant to conversion.

Methods

Bisulfite sequencing applies routine sequencing methods on bisulfite-treated genomic DNA to determine methylation status at CpG dinucleotides. Other non-methylation-specific strategies are also employed to interrogate the methylation at specific sites or at a genome-wide level. All strategies assume that bisulfite-induced conversion of unmethylated cytosines to uracil is complete, and serves as the basis of all subsequent techniques. Ideally, the method used would determine the methylation status separately for each allele. Alternative methods to bisulfite sequencing include Combined Bisulfite Restriction Analysis and methylated DNA immunoprecipitation (MeDIP).

The methodologies can be generally divided into strategies based on methylation-specific PCR (MSP) (Figure 4), and strategies employing primer extension reaction (PCR) performed under non-methylation-specific conditions (Figure 3). Microarray-based methods use PCR based on non-methylation-specific conditions also.
Microarray-based methods level methylation the specificity of the PCR for successfully the involved sequences. Quantitation is made in

The Methylation method is based on MSP, but provides a quantitative analysis using real-time PCR.

Pyrosequencing has also been used to analyze bisulfite-treated DNA without using methylation-specific PCR. Following PCR amplification, Pyrosequencing is used to determine the bisulfite-conversion sequence of specific CpG sites in the region. The ratio of C-to-T at individual sites can be determined quantitatively based on the amount of C and T incorporation during the sequence extension. The main limitation of this method is the cost of the technology. However, Pyrosequencing does well also for high throughput screening methods.

A further improvement to this technique was recently described by Wong et al., which uses allele-specific primers that incorporate single-nucleotide polymorphisms into the sequence of the sequencing primer, thus allowing for separate analysis of maternal and paternal alleles. This technique is of particular usefulness for genome imprinting analysis.

High-throughput analysis melting analysis (HRM)

A further method to differentiate converted from unconverted bisulfite-treated DNA is using high-resolution melting analysis, which is a real-time PCR-based technique initially designed to distinguish SNPs. The PCR amplicons are analyzed directly by temperature ramping and resulting fluorescence of intercalating dye during melting. The degree of methylation, as represented by the C-to-T content in the amplicon, can be determined by analyzing the fluorescence signal during melting.

Methylation-sensitive single nucleotide primer extension (MS-SnPE)

MS-SnPE employs the primer extension method initially designed for analyzing single-nucleotide polymorphisms. DNA is bisulfite-converted, and primer-specific primers are annealed to the sequence up to the base pair immediately before the CpG of interest. The primer is extended by two base pairs, one using DNA polymerase terminating dinucleoside monophosphates, and the ratio of C-to-T is determined quantitatively.

A number of methods can be used to determine the C/T ratio. At the beginning, MS-SnPE relied on radioactive dNTPs as the reporter of the primer extension. Fluorescence-based methods or Pyrosequencing can also be used. However, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis to differentiate between the two polymorphic primers by mass spectrometry can also be used, based on the GQ assay designed for SNP genotyping. A further improvement to this technique was recently described by Akiyama et al., which incorporates a fluorescence reporter probe.

Base-specific cleavage MALDI-TOF

A recently described method by Eishi et al. further takes advantage of bisulfite-conversions by adding a base-specific cleavage step to enhance the information gathered from the DNA. By cleaving the DNA at specific nucleotides, the PCR product can be cleaved at specific sites, allowing for high-throughput screening, for allowing for interpretation of numerous CpG sites in multiple tissues in a cost-effective manner.

Methylation-specific PCR (MSP)

This method uses bisulfite-treated DNA but avoids the need to sequence the area of interest. The bisulfite-treated DNA is denatured, and the "methylated" sites are converted to unmethylated cytosines using sodium bisulfite. The DNA is then treated with a restriction enzyme that cleaves specifically at methylated CpG sites. The resulting fragments are then separated by agarose gel electrophoresis, and the band intensity is measured by densitometry. This method allows for the quantification of methylation at individual CpG sites.

Further methodology using MSP amplified DNA analysis the products using melting curve analysis (MSP-MC). This method amplifies bisulfite-treated DNA with both methylated-specific and unmethylated-specific primers, and determines the quantitative ratio of the two products by comparing the different peaks generated in a melting curve analysis. A high-resolution melting analysis method that uses real-time quantitative and methylation analysis has been introduced, in particular, for sensitive detection of low-level methylation.
Microarray-based methods are a logical extension of the technologies available to analyze bisulfite-reated DNA to allow for genome-wide analysis of methylation. Oligonucleotide microarrays are designed using pairs of oligonucleotide hybridization probes targeting CpG sites of interest. One probe is complementary to the C-to-U-converted methylated sequence. The probes are also bisulfite-specific to prevent binding to DNA incompletely converted by bisulfite. The shine reaction is one such assay that applies the bisulfite sequencing technology on a microarray level to generate genome-wide methylation data.

Limitations

Incomplete conversion

Bisulfite sequencing relies on the conversion of every single unmethylated cytosine residue to uracil. If conversion is incomplete, the subsequent analysis will incorrectly interpret the unmethylated unmethylated cytosines as methylated cytosines, resulting in false-positive DNA methylation. Other concerns

This epigenomic information will be important in understanding how the measure of DNA methylation was feasible only using other techniques, such as Restriction landmark genomic scanning. The mapping of the human epigenome is seen as many scientists as the logical follow-up to the completion of the Human Genome Project. This epigenomic information will be important in understanding how the function of the genomic sequence is implemented and regulated. Since the epigenome is less stable than the genome, it is thought to be important in gene-environment interactions.

Epigenomic mapping is inherently more complex than genome sequencing, however. Since the epigenome is much more variable than the genome. While an individual has only one genome, small epigenomes varies with age, differs between tissue, is altered by environmental factors, and shows aberrant diseases. Such epigenomic markers, if present, are more specific for cells, tissues, and disease states, would yield valuable information on the normal function of epigenomic markers as well as the mechanisms leading to aging and disease.

Direct benefits of epigenomic mapping include probable advances in cloning technology. It is believed that failures to produce cloned animals with normal viability and wean from inappropriate parameters of epigenomic markers. Also, aberrant methylation patterns are linked to the development and progression of cancer, which may be an early indicator of cancer in high-risk individuals, thus enabling early intervention.

Applications: genome-wide methylation analysis

The advances in bisulfite sequencing have led to the possibility of applying them at a genome-scale level, where, previously, global measures of DNA methylation was feasible only using other techniques, such as Restriction landmark genomic scanning. The mapping of the human epigenome is seen as many scientists as the logical follow-up to the completion of the Human Genome Project. This epigenomic information will be important in understanding how the function of the genomic sequence is implemented and regulated. Since the epigenome is less stable than the genome, it is thought to be important in gene-environment interactions.

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Bisulfite conversion protocol

Human Epigenome Project (HEP) - Data

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Figure 2: Outline of the chemical reaction that underlies the bisulfite mediated conversion of cytosine to uracil.

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