Introduction to EpiTYPER for Quantitative DNA Methylation Analysis Using the MassARRAY® System

Mathias Ehrich, Devan Correll, and Dirk van den Boom

Introduction

Genetic information is not merely contained in the arrangement of the four nucleotide bases, but also in the covalent addition of methyl groups to cytosine within CpG dinucleotides. Methylation and related chromatin changes are important processes in the regulation of gene expression. The relevance of DNA methylation has been demonstrated in mammalian development, imprinting and X-chromosome inactivation\(^1\), suppression of parasitic DNA\(^2\), and various cancer types\(^3\)\(^\text{-}^6\). The ability to detect and to quantify methylation is particularly important to the field of cancer diagnostics. Changes in the methylation status of DNA have the potential to serve as an early detection marker for malignancies.

In the field of cancer research there is a need for an efficient method enabling quantitative DNA methylation analysis for a broad throughput range. Existing methods are limited; they are often too laborious for high throughput or inadequate for quantifying methylation. This article demonstrates that EpiTYPER is able to overcome the limitations apparent in existing methods. (See Figure 1.)

EpiTYPER is a tool for the discovery and quantitative analysis of DNA methylation that uses base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) introduced previously for SNP discovery\(^7\)\(^\text{-}^9\). Using the speed and accuracy of the MassARRAY® system, this method is ideal for discovery of methylation, for discrimination between methylated and non-methylated samples, and for quantifying the methylation levels of DNA\(^11\).

![Genomic DNA and EpiTYPER Mass Spectra](image)

The blue-colored genomic DNA represents a methylated sample. Methylated CpG sites are represented by black circles. The red-colored genomic DNA represents a normal sample. The mass spectra represent typical signal patterns seen for these types of samples after they have been processed using EpiTYPER. The dotted lines mark the signals representing non-methylated template DNA.

Figure 1. Example of EpiTYPER mass spectra indicating methylation levels in cancer and normal samples
Applying the EpiTYPER Assay

Overview

EpiTYPER is a bisulfite-treatment-based method for detection and quantitation of DNA methylation. Bisulfite treatment of genomic DNA converts non-methylated cytosine into uracil while methylated cytosine remains unchanged. Bisulfite treatment produces methylation-dependent sequence variations of C to T in the amplification products. These C/T variations appear as G/A variations in the cleavage products generated from the reverse strand by base-specific cleavage. These G/A variations result in a mass difference of 16 Da per CpG site, which is easily detected by the MassARRAY® system. In the mass spectrum, the relative amount of methylation can be calculated by comparing the signal intensity between the mass signals of methylated and non-methylated template.

The method starts with bisulfite treatment of genomic DNA, followed by PCR amplification in which a T7-promoter tag is introduced. The PCR primers should be designed to yield a product within a 200-600 bp range. (Bisulfite treatment often limits the success of PCR when longer amplicons are used.) The significant advantage of this method is that the PCR primers are independent of the methylation state of the genomic DNA, meaning they bind to both methylated and non-methylated template (as opposed to methylation-specific primers). Only two primers are needed to screen for methylation changes within a region of several hundred bases in a single experiment. Next, in vitro RNA transcription is performed on the reverse strand, followed by base-specific cleavage. In the cleavage reaction, the reverse strand is cleaved by RNase A at specific bases (U or C). Cleavage products are generated for the reverse transcription reactions for both U (T) and C in separate reactions. (T is used from this point forward, since it is found in DNA. Cleavage, however, actually happens at U in an RNA molecule.) MALDI-TOF MS analyzes the cleavage products, and a distinct signal pair pattern results from the methylated and non-methylated template DNA. (See Figure 2.)

EpiTYPER generates quantitative results for each cleavage product analyzed. Each cleavage product encloses either one CpG site or an aggregate of multiple CpG sites. An analyzed unit containing one or multiple CpG sites is called a “CpG unit”. For both T and C reactions, the resulting cleavage products have the same length and differ only in their nucleotide composition. Using this approach, EpiTYPER was applied to a model system using a CpG island in the IGF2/H19 region. The resulting mass signal pattern represented hemi-methylated DNA, and the signal intensity ratio of methylated to non-methylated signals was equivalent to 0.5, as expected. The standard deviation in quantitation is 5%. (See Figure 5 on page 4.)

Figure 2. Overview of EpiTYPER process
Mass Spectrum Reveals Methylation Patterns

As mentioned earlier, bisulfite treatment introduces a C to T shift on the forward strand. This variation leads to a G to A shift on the reverse strand, which is represented in the mass spectrum by signal pairs separated by 16 Da (or multiples thereof when multiple fragments are enclosed). In the resulting mass spectrum, there are distinct signals illustrating non-methylated and methylated template DNA. (See Figure 5.) Depending on the sequence of the target region and the distribution of CpGs, the mass spectrum may contain multiple signal pairs of cleavage products. These signal pairs can be used to determine the ratio of non-methylated to methylated DNA.

When a simulation was run for all 27,000 known CpG sites in the human genome, the EpiTYPER method was able to quantify approximately 90% of the CpG sites using two cleavage reactions. (Where CpG islands were longer than 500 bp, a 500 bp region was randomly selected for simulation.)

Information presented in the mass spectrum can be used to assess the degree of methylation for each CpG unit independently or to estimate the average methylation for the entire target region. Using this method, both hypermethylation and hypomethylation can be detected in samples. The MassARRAY® system is able to detect the methylation level in a mixture as low as 5%.

Figure 3. Spectra for hemi-methylated DNA, where relative methylation levels are 50%, as expected

Figure 4. EpiGram Tab Pane

Example of mass spectra generated by T-reverse cleavage reaction of the IGF2/H19 region. Arrows mark the position of mass signals representing non-methylated (NM) and methylated (M) CpGs. Signals with 16 Da shift (or a multiple thereof) represent methylation events.

Silent signals (S) are not affected by methylation but may overlay other signals. Their signal intensity changes can still be used to quantitate methylation.

This pane provides graphical representations of the CpG Sites within the selected amplicon. Each is color-coded to the degree of methylation shown in the methylation tab pane to provide a quick, reliable comparison between samples and CpG Sites.
Figure 5. Results from a mixture experiment are shown above. It is an example illustration taken from a “zoomed in” view of a spectrum generated in EpiTYPER. Figure 5A is an example of mass signal pairs for single CpG unit showing methylation ratios. Figure 5B is a graph of relative methylation levels derived from the same mixture experiment.
Large-Scale Application of EpiTYPER

In a study conducted with the Roy Castle International Centre for Lung Cancer Research, University of Liverpool, a large-scale application of EpiTYPER was used to characterize DNA methylation in 48 non-small cell lung cancer (NSCLC) patients. Forty-seven genes and their promoter regions were selected from literature and publicly available gene expression data. A total of 1,425 CpG sites were quantitatively analyzed for DNA methylation. Two-way hierarchical clustering was performed based on the quantitative methylation results. This clustering was used to identify differentially methylated regions, which contribute to the disease phenotype. The NSCLC study revealed significantly different methylation patterns between normal and tumor tissue.

EpiTYPER data was collected for 599 CpG units. The relative methylation levels were calculated by comparing the signal intensity for the mass signals from methylated and non-methylated template DNA. The relative methylation data was filtered for quality (data must be available for more than 90% of all samples) and for variance (variance >0.01). The resulting subset of 248 CpG units was used in a two-way hierarchical clustering analysis. This analysis identified two main clusters that are mostly populated by either tumor or normal samples, indicating that EpiTYPER is useful in the precise characterization of epigenetic changes underlying disease phenotypes. See Figure 6.

Figure 6. Cluster of relative methylation from NSCLC and normal samples

Highlights

EpiTYPER provides the following benefits:

- Rapid, automated discovery of multiple methylated CpG positions in regions of 200-600 bp
- Simplified assay design; no extension primers needed
- No need for cloning of PCR products
- Quantitative assessment of the degree of methylation
- Simple identification of hypomethylation and hyper-methylation
- Detection of methylation levels as low as 5% in sample mixtures
- Results may be obtained from various sample types, including frozen tissue, mouth swabs, and paraffin-embedded tissue
- High precision and reproducibility
- Scalable throughput on an established MassARRAY® system
- Gene expression, SNP discovery, genotyping, and allelotyping studies may be run on same platform
**Methods**

**Primer Design**

Primer are designed as illustrated in Figure 7. The recommended size range for PCR amplicons is 200-600 bp. (Longer amplicons were also analyzed, but bisulfite treatment limits the success of PCR.) Primers were designed using MethPrimer ([http://www.urogene.org/methprimer/](http://www.urogene.org/methprimer/)).

Design the following primer systems for use in methylation analysis:

- T7-promoter tagged reverse primer to obtain an appropriate product for *in vitro* transcription. An 8 bp insert is included to prevent abortive cycling.

- 10mer-tagged forward primer to balance the PCR.

**Figure 7. PCR primers for reverse transcription**

**Bisulfite Treatment**

To run bisulfite conversion treatment, use either the EZ-96 DNA Methylation Kit or the EZ DNA Methylation Kit from Zymo Research ([http://www.zymoresearch.com](http://www.zymoresearch.com)).

After this protocol’s initial incubation, cycle as follows:

- 45 cycles:
  - 95°C for 30 minutes,
  - 50°C for 15 minutes.

**Step 1: Amplification**

Amplify 1 μL DNA in a 5 μL total volume using a 384-microtiter format. (Use 1.00 μL of at least 10 ng/μL DNA for a final concentration of 2 ng/μL per reaction.) Each PCR will be split into two cleavage reactions (T Cleavage reaction and C Cleavage reaction).

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<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration for Single Reaction</th>
<th>Volume for Single Reaction</th>
<th>Volume for 384-Well Microplate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>dd H2O</td>
<td>N/A</td>
<td>1.42 μL</td>
<td>806.6 μL</td>
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<tr>
<td>10X Hot Star Buffer (QIAGEN), containing 15 mM MgCl2, Tris-Cl, KCl, (NH4)2SO4, pH 8.7 (20°C)</td>
<td>1x</td>
<td>0.50 μL</td>
<td>284.0 μL</td>
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<tr>
<td>dNTP mix, 25 mM each</td>
<td></td>
<td>0.04 μL</td>
<td>22.7 μL</td>
</tr>
<tr>
<td>5 U/μL Hot Star Taq (QIAGEN)</td>
<td>0.2 unit</td>
<td>0.04 μL</td>
<td>22.7 μL</td>
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**Table:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Final Concentration for Single Reaction</th>
<th>Volume for Single Reaction</th>
<th>Volume for 384-Well Microplate‡</th>
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</thead>
<tbody>
<tr>
<td>1 μM forward primer</td>
<td>200 nM (1 pmol)</td>
<td>1.00 μL</td>
<td>variable‡</td>
</tr>
<tr>
<td>1 μM reverse primer</td>
<td>200 nM (1 pmol)</td>
<td>1.00 μL</td>
<td>variable‡</td>
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**DNA**

<table>
<thead>
<tr>
<th>Total Volume</th>
<th></th>
<th></th>
<th>variable†</th>
</tr>
</thead>
</table>

† Volumes for a 384-well microplate include 48% overhang to account for possible pipetting loss
‡ Dependent on the combination of samples and amplicons used per plate

Seal the plates and cycle as follows:

- 94°C for 15 minutes
- 45 cycles:
  - 94°C for 20 seconds,
  - 56°C for 30 seconds*
  - 72°C for 1 minute
- 72°C for 3 minutes

*Adjust annealing temperature according to melting temperature of the primers.

**Note:** If you have questions about the suitability of your genomic DNA or performing the bisulfite treatment, contact Sequenom customer support at 1 877 4 GENOME (toll free within the U.S.) or at helpdesk@sequenom.com.
**Step 2: Dephosphorylation**

Add 2 μL of Shrimp Alkaline Phosphatase (SAP) enzyme to each 5 μL PCR to dephosphorylate unincorporated dNTPs from the PCR. Incubate the plates for 20 minutes at 37º C. Then, incubate at 85º C for 5 minutes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for Single Reaction</th>
<th>Volume for 384-Well Microtiter Plate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free ddH₂O</td>
<td>1.70 μL</td>
<td>901 μL</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase (SAP)</td>
<td>0.30 μL</td>
<td>159 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>2.00 μL</strong></td>
<td><strong>1060 μL</strong></td>
</tr>
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</table>

† Volumes for a 384-well microtiter plate include 38% overhang to account for possible pipetting loss.

**Step 3: In Vitro Transcription and RNase A Cleavage**

Prepare transcription/RNase A cocktail for each cleavage reaction (T and C). For standard setup, prepare one transcription/RNase A cocktail per plate. Add 5 μL of transcription/RNase A cocktail and 2 μL of PCR/SAP sample into a new, uncycled microtiter plate. Centrifuge the plates for one minute. Then, incubate the plates at 37º C for 3 hours.

<table>
<thead>
<tr>
<th>T Cleavage Transcription/RNase A Cocktail</th>
<th>Final Concentration for Single Reaction (in 7 μL)</th>
<th>Volume for Single Reaction</th>
<th>Volume for One Sample Microtiter Plate†</th>
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</thead>
<tbody>
<tr>
<td>RNase-free ddH₂O</td>
<td>N/A</td>
<td>3.15 μL</td>
<td>1572.5 μL</td>
</tr>
<tr>
<td>5x T7 Polymerase Buffer</td>
<td>0.64 x</td>
<td>0.89 μL</td>
<td>444.3 μL</td>
</tr>
<tr>
<td>T Cleavage Mix</td>
<td>N/A</td>
<td>0.24 μL</td>
<td>119.8 μL</td>
</tr>
<tr>
<td>DTT, 100mM</td>
<td>3.14 mM</td>
<td>0.22 μL</td>
<td>109.8 μL</td>
</tr>
<tr>
<td>T7 RNA &amp; DNA Polymerase</td>
<td>22 units/reaction</td>
<td>0.44 μL</td>
<td>219.6 μL</td>
</tr>
<tr>
<td>RNase A</td>
<td>0.09 mg/mL</td>
<td>0.06 μL</td>
<td>30.0 μL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td><strong>5.00 μL</strong></td>
<td><strong>2496 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

† Volumes are for a 384-well microtiter plate and include approximately 33% overhang to account for possible pipetting loss.

**Step 4: Sample Conditioning**

Add 20 μL of ddH₂O to each sample within the 384-well plate. Add 6 mg of Clean Resin to each well using the resin plate. Rotate for 10 minutes and spin down for 5 minutes at 3,200 x g.

**Note:** To avoid pH shifts, always add ddH₂O first before adding the Clean Resin.

**Step 5: Sample Transfer**

Dispense 10-15 nL of EpiTYPER reaction product onto a 384-element SpectroCHIP® bioarray.

**Step 6: Sample Analysis**

Acquire spectra from the two cleavage reactions using the MassARRAY® system.

**Step 7: Analysis Software**

Results can be analyzed using the EpiTYPER software. This software is not yet available for purchase. Commercial release of EpiTYPER software is scheduled for Q1 2006.

**EpiTYPER Requirements**

**Hardware and Software**

- MassARRAY® Analyzer (Part Number 00450) or
- MassARRAY® Analyzer Compact (Part Number 10920)

- EpiTYPER software (Part Number to be determined)

**Consumables**

- MassCLEAVE Kit (Part Number 10129)

Contact your SEQUENOM sales or customer support representative for information.
References


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