Chapter 18

Methylation SNaPshot: A Method for the Quantification of Site-Specific DNA Methylation Levels

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Abstract

As the role for epigenetic signals in genome regulation becomes increasingly understood, the ability to accurately measure levels of DNA methylation at individual cytosines throughout the genome is becoming increasingly important. In contrast to traditional methods for the quantification of cytosine methylation, such as cloning and sequencing of PCR fragments amplified from sodium bisulfite-modified DNA, recent developments have created a fast and effective alternative called methylation-sensitive single nucleotide primer extension (Ms-SNuPE). The following protocol outlines the steps necessary to design and perform Ms-SNuPE experiments using the SNaPshot® chemistry and associated capillary electrophoresis platforms available through Applied Biosystems.

Key words: Epigenetics, DNA methylation, Ms-SNuPE, SNaPshot, sodium bisulfite.

1. Introduction

Methylation of DNA at CpG dinucleotides has been recognized as a key regulator of critical genomic functions, such as transcriptional regulation, silencing of repetitive DNA, and formation and stabilization of heterochromatic regions of the genome (e.g., centromeric and pericentromeric regions). DNA methylation plays a critical role in normal human development, is a key regulator of genomic imprinting, and has been shown to modulate some gene expression responses to environmental stimuli, in some cases by changing the methylation status of a single CpG *(1)*. DNA methylation aberrations have been implicated in numerous rare developmental disorders such as Fragile X syndrome *(2)*, Beckwith–Wiedemann syndrome *(3)*, and Prader–Willi and

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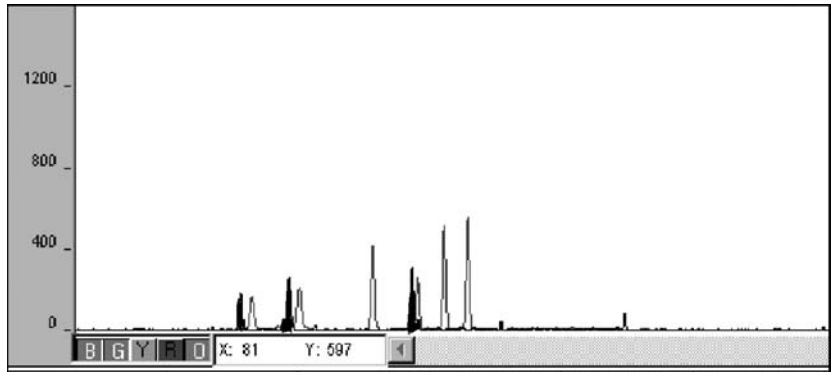
Angelman syndrome, among others *(4)*, as well as in various forms of cancer *(5–7)*. DNA methylation is therefore a prime target for study and represents a promising target for diagnostic and therapeutic advances in the future.

The "gold standard" method for the quantification of DNA methylation is based on the sodium bisulfite-based fine mapping of individual CpG dinucleotides, the preferred target of DNA methylation in the human genome. Sodium bisulfite modification deaminates nonmethylated cytosines (C) to uracils, which are subsequently amplified as thymines (T) by polymerase chain reaction (PCR). The end result is a C/T polymorphism in the sequence whose ratio is relative to the original levels of methylated to nonmethylated cytosines. Traditional methods to interrogate these sequence differences involve cloning and sequencing of a large number of sequences per individual to get an accurate quantification of the methylation polymorphism *(8, 9*, **Chapter 14***)*. However, such an approach is very labor intensive and time consuming. In recent years, efficient methods based on single nucleotide primer extension reactions have been developed to quickly and accurately quantify levels of DNA methylation. As indicated by the name, the reaction is based on repeated annealing of a primer exactly one base pair upstream of a target CpG and extending the primer by the incorporation of a single fluorescent dideoxynucleotide. Subsequently, the proportion of the incorporated fluorescent signals that represent C or T can be measured by electrophoresis. As this principle of allele differentiation has been widely applied to single nucleotide polymorphisms (SNPs), some researchers have coined the term methylation-sensitive single nucleotide primer extension (Ms-SNuPE). The method is not limited to one analysis platform, with some groups using radioactively labeled dNTPs or mass spectrometry for quantification *(10, 11*, **Chapter 16***)*, our laboratory uses the ABI SNaPshot reaction platform to perform Ms-SNuPE. In the ABI SNaPshot reaction, multiplexed primers of variable size, targeting different CpG dinucleotides, are cycled with fluorescent dideoxynucleotide terminators that halt the reaction after incorporation. Upon capillary electrophoresis, the different-sized primers migrate at different rates through the polymer matrix, allowing the proportions of incorporated fluorescent signal to be correlated with each target region (**Fig. 18.1**). As the proportion of fluorescent signal is representative of the original proportions of sodium bisulfite-converted C and T, a quantitative measure of DNA methylation is obtained at various positions.

Regions of interest to epigenetic research often have a high GC content, such as the CpG island regulatory elements that are often associated with housekeeping gene promoters. Therefore, strategies for Ms-SNuPE primer design are of particular importance, especially avoiding the incorporation of potentially



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 1	5.71	24.01	190	1974	2141
Y, 2	5.96	31.01	259	2362	2236
Y, 3	6.61	48.96	315	2972	2480
Y, 4	7.74	79.92	92	317	2901
Y, 5	8.79	109.92	63	247	3296
Y, 6	9.13	119.92	66	260	3422
R, 1	5.78	25.66	170	1768	2166
R, 2	6.02	32.61	217	2560	2256
R, 3	6.40	43.27	426	3912	2399
R, 4	6.65	49.86	263	2300	2493
R, 5	6.80	53.71	519	4512	2549
R, 6	6.93	57.10	567	4795	2598

Fig. 18.1. Example output of a multiplexed SNaPshot reaction interrogating six separate CpG dinucleotide positions. Shaded peaks represent the proportion of C while unshaded ones represent T. Positions 1, 2, and 4 display approximately 50% methylation, while the remaining CpG positions are entirely unmethylated. Positional coordinates for each peak as well as the peak height information necessary to calculate the methylation percentage for each CpG are displayed below the peak readout.

polymorphic positions into the Ms-SNuPE primer annealing region. Mismatches in the primer annealing region can introduce large biasing effects to the measured levels of DNA methylation *(12)*. Primer design is, therefore, one of the key aspects of this protocol, in addition to performing sodium bisulfite modification, followed by the Ms-SNuPE reaction and quantification. These four steps will be covered in detail in the sections to follow.

2. Materials

2.1. Post Sodium Bisulfite Modification PCR Primer Design

1. Computer with Internet connection.
2. Account with an oligonucleotide synthesis company for ordering primers.

2.2. Ms-SNuPE Primer Design

1. Same as **Section 2.1**.

2.3. Sodium Bisulfite Modification

1. Sterile water, preferably freshly degassed under a vacuum (*see* **Note 1**).

2. Fresh 3 *M* NaOH solution. Dissolve 3 g NaOH pellets in 25 mL of the degassed water.
3. Fresh 0.1 *M* NaOH solution made from a dilution of above.
4. Fresh hydroquinone solution. Dissolve 0.22 g hydroquinone (Sigma) in 10 mL degassed water. Keep this solution shielded from light.
5. Saturated sodium bisulfite solution. Bring 10.8 g sodium bisulfite (Sigma) to 16 mL final volume in preheated degassed water (55°C). Invert to mix until solution is fully saturated. Add 2.6 mL 3 *M* NaOH solution and 1.0 mL hydroquinone solution. Mix well (*see* **Note 2**).
6. Microcon YM-50 columns (Millipore) or (for high-throughput) Montage PCR96 96-well filtration plates (Millipore) (*see* **Note 3**).
7. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.5.

2.4. PCR

1. HotStar Taq Master Mix (Qiagen).
2. Forward and reverse PCR primers, 20 μM in TE buffer.

2.5. Gel Electrophoresis

1. 1 × TBE Buffer: 89 mM Tris–boric acid, 2 mM EDTA.
2. 5 mg/mL Ethidiumbromide.
3. 100 bp DNA ladder (50 ng/μL).

2.6. PCR Clean-Up

1. Qiagen Minelute gel extraction kit (Qiagen).

2.6.1. Option 1: Qiagen Gel Extraction

2.6.2. Option 2: Exo I/SAP Digestion

1. Exonuclease I (20 U/μL) and corresponding 10 × reaction buffer.
2. Shrimp Alkaline Phosphatase (10 U/μL) and corresponding 10 × reaction buffer.

2.7. The SNaPshot Reaction

1. SNaPshot Multiplex Reaction Kit (Applied Biosystems).
2. 5 × Sequencing buffer (Applied Biosystems).
3. Ms-SNuPE primers, resuspended in TE Buffer to a final concentration of 20 μM.

2.8. Removal of Unincorporated ddNTPs

1. Calf intestinal phosphatase (CIP) (10 U/μL) and corresponding 10 × reaction buffer.

2.9. Capillary Electrophoresis

1. ABI 3100 Avante Genetic Analyzer (Applied Biosystems).
2. Genescan 3.1 software (Applied Biosystems).
3. Pop4 polymer (Applied Biosystems).
4. 36-cm array (Applied Biosystems).
5. 10 × EDTA buffer (Applied Biosystems).
6. Optical plate with denaturation cover and septum (Applied Biosystems).

7. GS120 LIZ size standard (Applied Biosystems).
8. DS-02 Matrix standard kit (Applied Biosystems).
9. Hi Di formamide (Applied Biosystems).

3. Methods

3.1. Post Sodium Bisulfite Modification PCR Primer Design

One of the most critical steps for successfully performing Ms-SNuPE reactions is the assay design stage concerning the design of both bisulfite-PCR and Ms-SNuPE primers. More specifically, it is critical that the primers designed to produce the post sodium bisulfite-modified PCR amplicon do amplify efficiently and specifically. Obtaining a good yield from a post-bisulfite-treatment PCR can be a challenge due to the high (~90%) degradation of genomic DNA that occurs during bisulfite treatment *(13)*. Bisulfite treatment can also affect the specificity of some PCR primers because the degenerated genetic code (all unmethylated cytosines converted to thymines) reduces the sequence complexity of some regions and thus produces a risk for nonspecific PCR amplification. Finally, because the methylation status of any CpG dinucleotide is not known a priori, PCR primers should avoid internal CpG incorporation where possible to avoid mispriming. The following steps should be taken to improve the chances of successful post-bisulfite amplification.

1. Select a region of interest within the genome around which to design primers. Be sure to avoid designing primers in repetitive regions of the genome, which can be identified using Repeat Masker in the UCSC genome browser (http://genome.ucsc.edu). The amplification efficiency of the reaction is often dependent on amplicon size, such that a smaller amplicon tends to amplify better. Amplicons less than 300 bp in length can often be amplified with a single PCR. For larger PCR amplicons (>400 bp), sometimes a nested or semi-nested PCR strategy is required. We have not exceeded 600 bp. The optimal amplicon size is, of course, a factor of the region of interest. Once a PCR amplicon has been produced, Ms-SNuPE can be performed on any CpG site within the amplicon and so, depending on the target CpGs, the ideal amplicon should be designed on a case-by-case basis.
2. If the region of interest is a repetitive region, design at least one of the primers in a unique region of the genome to target that specific repetitive element. If the repetitive region is large and if placing a primer in a unique genomic region would cause the amplicon of interest to exceed ~600 bp, a useful strategy is to perform a nested or semi-nested PCR, ensuring that one of the first set of primers is in a unique region of the genome

for the first amplification. The subsequent PCR in the second step can then target your amplicon sequence of interest.

3. Once the general target region has been determined, begin designing PCR primers for the region. Online PCR design software such as MethPrimer (http://www.urogene.org/methprimer/index1.html) performs well and allows the user to specify various parameters such as amplicon size, excluded regions, and primer TM, among others, and returns converted (specific to bisulfite-modified sequence) primer sequences with the specified parameters *(14)*. MethPrimer does not require the electronic conversion of the sequence of interest to a post-bisulfite sequence. However, if the software fails to produce primers for a specific region, it is possible to design primers by hand or use alternative primer design software. In such cases, it will be necessary to manually convert your sequence of interest into a post-bisulfite-modified sequence by converting all "C" to "T" prior to designing primers. When designing primers manually, it is best to select a region that contains some positions that contain cytosines in a non-CpG context to specifically amplify completely converted sequences and avoid homopolymers of any nucleotide that might bind nonspecifically or form hairpin structures (*see* **Note 4**).

3.2. Ms-SNuPE Primer Design

All Ms-SNuPE primers should be designed so that the 3′ end of the primer terminates exactly 1 bp upstream (5′) of the target C of the CpG dinucleotide. Because Ms-SNuPE primers are distinguished by size, it is important to have primers synthesized and purified by HPLC to limit primer size variants. Primers can be designed in either the forward orientation (complementary to the antisense strand) or the reverse orientation (complementary to the sense strand) (*see* **Note 5**). In this way, the design of Ms-SNuPE primers is relatively easy; however, designing the most efficient assay will be dependent on the number of target CpGs and the surrounding CpG density. These factors will affect both the multiplexing capability of the assay and the subsequent clarity of the final electrophoretic profile (**Fig. 18.1**). At this point, it is useful to distinguish between two primer design scenarios that will affect the ability to multiplex the reaction, these being whether there are CpG dinucleotides within the primer binding sequence or not.

3.2.1. Design of Multiplex Assays (When there are "no" CpG dinucleotides in the Ms-SNuPE primer annealing region)

1. **Primer Size:** If there are no CpG dinucleotides in the primer binding sequence, multiplexing (performing more than one single nucleotide primer extension reaction in the same tube) is possible (*see* **Note 6**). When considering the design of a multiplexed assay, the cost of primer synthesis should be weighed against the cost of running reactions separately as for the

electrophoretic separation of the various target CpG dinucleotides, the primers to be multiplexed must differ in length by at least 8 bp and 4 bp below and above a total primer length of 40 bp, respectively. For example, if a second Ms-SNuPE primer is multiplexed with an existing 20 bp primer, the second primer should be at least 28 bp while if the original was 40 bp, the second could be either 32 bp or 44 bp. In our experience, 15–17 bp is the lower limit for primer size, and in an ideal situation of 10 multiplexed reactions, the longest primer would therefore be around 65 bp. Of course, the sequence features of your target oligonucleotide of interest may limit the total number of possible primers to multiplex at once.

2. **Primer TM:** All primers to be multiplexed together should be designed with similar TM. The suggested SNaPshot reaction cycling conditions have an annealing temperature of 50°C and so primers should be designed with a TM of 50°C. The annealing temperature in the cycling protocol can be manipulated to better suit the primers. However, to ensure equal performance in the cycling reaction, all primers should be designed with similar TM (± 3°C). Because of the necessity to vary primer length while keeping the same range of primer TM, primer lengths should be varied by adding noncomplementary tails to the 5′ end of each primer, with primer TM being calculated only for that section of the primer that is complementary to the target amplicon. The sequence 5′-GACT-3′ × N can be used as a nonbinding tail as it does not form hairpin loops (*see* **Note 7**).
3. **Primer Orientation:** Ms-SNuPE Primers complementary to the T-rich sense and A-rich antisense strands, respectively, must not be multiplexed together to avoid primer dimer formation.

*3.2.2. Design of Single Assays (*When there "are" CpG dinucleotides in the Ms-SNuPE primer annealing region*)*

In general, the presence of one or more CpG dinucleotides within the Ms-SNuPE primer binding region requires an additional step in the primer design (*see* **Note 8**). This step is simply to design degenerative or "wobbling" bases at the C position of any upstream CpG dinucleotides within the primer binding region. If designing primers complementary to the antisense strand (Forward Primers), primers should be designed with Y's in place of C's, while reverse strand primers should be designed with R's at the potentially polymorphic positions. The incorporation of wobbling bases in the primer makes multiplexing primers infeasible in terms of downstream data interpretation (*see* **Note 9**).

3.3. Sodium Bisulfite Modification

1. Adjust volume of the DNA sample (50 ng–2 μg) to 10 μL (*see* **Note 10**).
2. Transfer DNA sample(s) to PCR tubes (or 96-well plate for high-throughput processing). Add 1.1 μL of freshly prepared 3 M NaOH solution. Centrifuge and seal the tubes (or plate).

3. Place in a thermocycler for 20 min at 42°C.
4. Spin down tubes/plate to catch condensation and carefully open seal. Add 120 μL of fresh sodium bisulfite solution, seal plate/tube with a fresh lid, invert a few times to mix, and then spin-down.
5. Place in a thermocycler for 4–5 h at 55°C (*see* **Note 11**).
6. Remove from thermocycler, spin down, and carefully remove lid.
7. Add 100 μL of sterile water.
8. Transfer each sample to a Microcon YM-50 column or (for high-throughput) the corresponding well in a Montage PCR96 96-well filtration plate.
9. Draw solution through filtration matrix by either centrifugation at maximum speed (Microcon YM-50 column) or vacuum (Montage PCR96 96-well filtration plate), until wells are visibly empty of solution (~4–5 min). DNA remains on the matrix.
10. Desalt DNA by adding 175 μL of sterile water to each well and drawing the solution through the matrix (via centrifugation or vacuum) as before. Repeat this step twice more.
11. Desulfonate by adding 175 μL of fresh 0.1 *M* NaOH. Draw solution through the matrix (via centrifugation or vacuum).
12. Perform a final washing step by drawing 175 μL of sterile water through the matrix.
13. Recover DNA by adding 50 μL of TE Buffer and incubation for 2 min.
14. If using Microcon YM-50 columns, carefully vortex for additional 30 s. Uncap Microcon unit, separate sample reservoir from filtrate cup, and place sample reservoir upside down into a new vial. Spin for 3 min at 1780*g* in invert spin mode to elute DNA. If using Montage PCR96 96-well filtration plate, use a plate shaker to release DNA from filtration matrix for 10 min at 500 rpm. Remove eluted DNA solution from individual wells using a pipette and filter tips.
15. Remove an aliquot for whole genome amplification if needed *(15*, **Chapter 27***)*. Otherwise, store bisulfite-treated DNA at −20°C (or −80°C for long-term storage).

3.4. Post Sodium Bisulfite Modification PCR

1. In PCR strip tubes, add approximately 4 μL of the eluted sodium bisulfite-treated sample as template for PCR.
2. Add forward and reverse PCR primers to a final concentration of 0.5 μM in the final reaction (0.5 μL of each forward and reverse 20 μM PCR primer stock).
3. To this, add 10 μL of Qiagen HotStar Taq Master Mix, which contains all reagents necessary for PCR, and 5 μL of ddH_2O, bringing the final reaction volume to 20 μL.
4. Cycle in a thermocycler according to the following specifications (*see* **Note 12**):

95°C – 15 min
{95°C – 30 s, 50°C – 45 s, 72°C – 30 s} 40 cycles
72°C – 5 min
Cool to 4°C

3.5. Amplicon Evaluation

To insure that the target amplicon has been amplified, the PCR needs to be evaluated by agarose gel electrophoresis.

1. Make a 2% TBE agarose gel. First, add the volume of 1 × TBE buffer required to fill the electrophoresis tray to an Erlenmeyer flask followed by 2 × weight per volume of biotechnology grade agarose. For example, to make 100 mL of 2% TBE agarose gel, add 2 g of agarose to 100 mL of 1 × TBE.
2. Heat the mixture by microwaving for approximately 3 min until solid agarose is no longer visible.
3. Allow the gel to cool for a few minutes and stir in 1 μL per 100 mL of ethidiumbromide (5 mg/mL) to achieve a final concentration of 0.5 μg/mL in the solution (*see* **Note 13**). When the gel is cool, it can be poured into the gel tray equipped with loading combs and allowed to polymerize (*see* **Note 14**).
4. Run approximately 5 μL of the PCR product on a 2% TBE agarose gel against 3 μL of 50-ng/μL 100-bp ladder to determine if the band of interest was specifically amplified. If there is nonspecific amplification, but the band is present and clearly distinguishable at the correct size, the rest of the PCR product can be run on a second gel and a scalpel or razor used to excise the band. Subsequently extract the amplicon using Qiagen's Minelute Gel Extraction kit, after which the SNaPshot reaction can be performed (*see* **Note 15**) (PCR Clean Up Option 1). If a single PCR amplicon was produced for the desired fragment, use the more rapid treatment involving digestion with exonuclease 1 and shrimp alkaline phosphatase (Exo I/SAP, PCR Clean Up Option 2).

3.6. PCR Clean Up

3.6.1. Option 1: Qiagen Gel Extraction

1. Weigh the excised gel fragment within an Eppendorf tube first by zeroing an analytical balance on an empty Eppendorf and then weighing the tube containing gel.
2. To that tube, add 3 volumes per weight of buffer QG. For example, to 100 mg of gel, add 300 μL of buffer QG. Incubate at 50°C for approximately 10 min, vortexing every 2 min, until no visible agarose remains.
3. Add the contents to a provided microtube column and spin at high speed (18,890*g*) in a microcentrifuge for 1 min.
4. Discard the flow through, add 500 μL of QG to the column, and repeat the spinning procedure including removal of flow through.

5. Add 750 μL of buffer PE, spin at high speed for 1 min, remove the flow-through, and then repeat the spin, without adding any additional buffers, to dry the column in preparation for elution.
6. Remove the column and place it in a newly labeled Eppendorf tube. Add 10 μL of elution buffer EB to the center of the column matrix and let stand for 1 min. Spin for 1 min at high speed (*see* **Note 16**).
7. Repeat the elution procedure to increase the purification yield, ending with a final volume of ~20 μL.

3.6.2. PCR Clean Up Option 2: Exo I/SAP Digestion

Digestion with ExoI/SAP can be more cost-effective than column-purification techniques and faster when large numbers of amplicons are prepared for the SNaPshot reactions.

1. Make a master mix containing 2 μL of ExoI and 5 μL of SAP per 15 μL of PCR sample and one-tenth of the final volume of 10 × NEB buffer 4.
2. Incubate samples at 37°C for 1 h followed by 15 min at 75°C.

3.7. Performing SNaPshot Reaction Cycling

1. The ABI protocol guidelines suggest using between 0.01 pmol and 0.40 pmol of amplicon template per reaction (*see* **Note 17**).
2. In PCR strip tubes, add a master mix comprised of 2 μL of SNaPshot Multiplex Ready Reaction Mix, 3 μL of 5 × Sequencing Buffer, 1 μL of 2 μM Ms-SNuPE primer (0.2 μM in the final reaction), and template and adjust the volume to 10 μL with ddH_2O (*see* **Note 18**).
3. Seal the strip tubes and place them in a thermocycler (preferably, but not necessarily an ABI 9700 thermocycler (*see* **Note 19**)), perform 25 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 30 s, followed by a final cool down to 4°C (*see* **Note 20**).

3.8. Removal of Unincorporated ddNTPs

In order to remove downstream fluorescent noise in the data analysis stage created by residual ddNTP terminators, calf intestinal phosphatase CIP digestion is required.

1. Make a master mix by adding 1 μL of CIP to 9 μL of NEB buffer 3. Add 3 μL of the master mix to each SNaPshot reaction well.
2. In a thermocycler, incubate at 37°C for 1 h followed by 15 min at 75°C and then allow cooling to 4°C (*see* **Note 21**).

3.9. ABI Electrophoresis and Data Quantification

The following protocol assumes the use of an ABI 3100 Avante Genetic Analyzer using POP4 polymer, a 36-cm Array that has been loaded with the DS-02 Matrix Standards. It should be noted that the capillary electrophoresis for SNaPshot can be performed on other ABI platforms.

1. Make a master mix containing 9 μL of Hi Di Formamide and 0.5 μL of GS 120 LIZ size standard per sample. Pipette 9.5 μL of this master mix into an ABI optical plate.
2. To each of these wells, add 0.5 μL of the CIP digested SNaPshot reaction mixture (*see* **Note 22**). Load samples in the required order, that is, in columns from top to bottom (not in rows from left to right) for maximal efficiency of the ABI 3100 Avante genetic analyzer (*see* **Note 23**).
3. Place a clean rubber denaturation lid on the plate and denature the samples in a thermocycler for 5 min at 95°C followed by immediate placement into an ice bath for 2 min. Replace the denaturation lid with a clean septum prior to running on the ABI 3100 Avante genetic analyzer.
4. In the ABI data collection software, open a new plate editor, name the project, and select Genescan. Create a valid sample name in the plate-coordinate rows that correspond only to those wells in the optical plate that contain your sample. Select orange for the analysis color, 3100 Avante for the project selection, Dye Set E5 for the dye set, SNP36_POP4DefaultModule as the Run Module, and GS120Analysis.gps for the Analysis Module.
5. Place your optical plate in the correct orientation in the machine, link your plate, and run. The SNaPshot electrophoresis takes about 30 min to complete four samples, assuming the use of an array with four capillaries.
6. After the run is complete, open the Genescan 3.1 Analysis software and select "New Project".
7. In the project window, go to "Add Samples" and select the plate name in the data extractor folder where the data is stored.
8. Set the marker to the color orange, which corresponds to the fluorescent signal of the GS 120 LIZ size standard. Clicking on a sample name will open a window that will show the various traces as well as quantitative estimates of the peak heights and positions (*see* **Note 24**). Optimal peak heights by default are above 50 intensity units on the Y axis (*see* **Note 25**). Incorporation of different ddNTP terminators will result in polymorphic positions having two peaks at a given location; however, due to slight differences in elecrophoretic migration, these peaks (such as a *C* and *T* peak) should appear to be separated by a distance of approximately 1 bp.
9. In the cases where Ms-SNuPE primers were designed with no "wobbling" positions, determine the peak position that corresponds to this primer (*see* **Note 22**) in the data display and determine the percentage of cytosine methylation (C^{met}) according to the following formula:

$$\%C^{\text{met}} = 100x\frac{(\text{Peak Height}C)}{(\text{Peak Height}C + \text{Peak Height}T)}$$

10. In cases where Ms-SNuPE primers were designed with "wobbling" positions, the $\%C^{\text{met}}$ can be determined by the following formula:

$$\%C^{\text{met}} = 100x \frac{{}_n\Sigma_1(\text{Peak Height}\, C_1 \ldots C_n)}{({}_n\Sigma_1 \text{Peak Height}\, C_1 \ldots C_n + {}_n\Sigma_1 \text{ Peak Height}\, T_1 \ldots T_n)}$$

where, C_1 is the peak height of the first C peak and C_n is the last (*see* **Note 26**). For Ms-SNuPE primers designed in the reverse orientation, look for G (Blue) and A (Green) peaks in place of C (Black) and T (Red) peaks, respectively.

4. Notes

1. Oxygen in water can reduce the efficiency of sodium bisulfite conversion.
2. It is possible that not all the sodium bisulfite will dissolve. If any substrate remains, centrifuge the solution and use the supernatant.
3. Using the Montage PCR96 96-well filtration plate (Millipore) for high-throughput sample processing requires a suitable vacuum manifold.
4. If either of the PCR primers is designed directly adjacent to the C position in a CpG dinucleotide, this PCR primer can later be used as a Ms-SNuPE primer.
5. Keep in mind that depending on what strand was targeted by the post-bisulfite-modification PCR, only one strand C of a palindromic CG will have a potentially polymorphic position. Therefore, whether using forward and reverse Ms-SNuPE primers, the target C of a given CpG should be the same.
6. Our laboratory uses an ABI 3100 Avante electrophoresis platform using the recommended Pop 4 polymer matrix. We have multiplexed up to six reactions successfully. However, the ABI SNaPshot manual suggests an upward multiplexing capacity of 10 primers in a reaction.
7. Investigate visually the 5′ upstream region of the primer region and avoid complementarity. For example, if the "T" of the GACT does not match, but "GAC" does, the repeating pattern should be shifted to 5′-TGAC-3′, for example.
8. Because of the unknown methylation status of these CpGs a priori, the target amplicon could contain polymorphic sequence at these positions. Experiments in our laboratory demonstrated a strong bias of the measured methylation percentage in cases where there is a polymorphic position in the primer annealing region and that this bias increases as the polymorphic position approaches the 3′ end of the Ms-SNuPE primer *(12)*.

9. The perfectly complementary primer in the degenerative mixture will bind the target and accurately measure the methylation percentage at the target CpG. However, this polymorphic mixture of primers is likely to have different electrophoretic potential and thus will separate, sometimes causing multiple peaks to be observed *(12)*. Quantification is covered in the data interpretation section but the presence of multiple peaks for a single primer would make the distinction of multiplexed primer peaks difficult. Therefore, it is advised to run Ms-SNuPE primers containing degenerate positions separately.
10. In our experience, the optimal starting amount of genomic DNA is 250 ng–1 μg. Small amounts of DNA (e.g., 50 ng or below) may not provide enough useable template for direct use following sodium bisulfite treatment, but can be used for DNA methylation profiling following WGA of the sodium bisulfite-treated DNA (**Chapter 27**).
11. It can be beneficial to ramp the reaction up to 95°C for 1 min each hour to ensure that the DNA remains single stranded.
12. The following cycling conditions generally work well as a universal program for amplification. Further optimization of primer annealing temperature may be required if the amplification does not work. If possible, run a positive control of sodium bisulfite-modified template that has been known to work in the past to rule out a failed bisulfite reaction in the event of PCR failure.
13. Ethidiumbromide is light sensitive, store in a dark place. Take care when handling, it is highly toxic.
14. To save time, a larger volume of gel can be made and stored in a 50°C incubator so that it is ready for the pouring stage. This solution is stable for about a month before the Ethidiumbromide decomposes.
15. It is necessary to purify the post-sodium bisulfite amplicon that serves as template for the SNaPshot reaction. Residual dNTPs could incorporate in place of the fluorescent ddNTP terminators provided in the SNaPshot reaction kit and other residual reaction components such as remaining PCR primers might bind the amplicon and incorporate fluorescent signals.
16. To avoid the Eppendorf tube lids snapping off, place tubes to be eluted side by side in pairs, with the lids crossed and facing inward toward the center of the centrifuge rotor.
17. Simply adding 1–2 μL of purified PCR product to the reaction will generally work without further quantification and calculation.
18. The addition of 5 × Sequencing Buffer is not necessary, as the original protocol calls for 5 μL of SNaPshot Multiplex Ready Master Mix. However, this buffer allows the more

expensive reaction component to be diluted, and thus one can achieve significantly more reactions at lower cost.

19. When performing SNaPshot reaction cycling, a fast ramping speed is desirable for optimal results.
20. If low signals are observed for a given primer or sample, the amount of starting template can be increased or the annealing temperature for the cycling reaction can be adjusted. Ms-SNuPE primer amounts can also be adjusted up or down to modulate signal intensity. However, the amount of template can be a limiting factor in cases of low signal, especially in cases where the PCR amplicon was observed to be weak during gel electrophoresis.
21. SNaPshot reactions can be stored at this point overnight at 4°C or for longer periods at −20°C prior to performing capillary electrophoresis on the ABI 3100 genetic analyzer.
22. Up to 2 μL each of SNaPshot mix and GS 120 LIZ size marker can be added to increase signal intensity as necessary, adding Hi Di Formamide to a final volume of 10 μL.
23. Because of the orientation of the ABI optical plate in the genetic analyzer, the capillary is oriented from top to bottom and thus will draw samples most efficiently if samples are loaded accordingly.
24. Peak positions are relative to the sizes of the peaks of the GS 120 LIZ size marker. The sizes are best thought of as relative sizes in that, for two Ms-SNuPE primers with expected sizes of 20 bp and 28 bp, these may appear as peaks at 25 bp and 37 bp. However, their relative sizes will identify which peaks correspond to which primer and thus target CpG.
25. There is no specified upper limit to the peak height. However, peaks should have a clear defined tip, not a flattening which signifies saturation of the fluorescent signal. When there is a flattened peak top, the data is no longer quantitative.
26. Experiments in our laboratory have demonstrated that this method of quantification is accurate within 5% of the true DNA methylation level as measured by sodium bisulfite-based cloning and sequencing *(12)*.

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