

Bisulfite Pyrosequencing

Christopher F. Bassil, Zhiqing Huang, and Susan K. Murphy

Abstract

Bisulfite pyrosequencing is a sequencing-by-synthesis method used to quantitatively determine the methylation of individual CG cytosines from PCR amplicons of a region up to 115 bases in length. The procedure relies on prior bisulfite conversion of all potentially methylated CG cytosines to either cytosine (methylated) or thymine (unmethylated) and involves the stepwise incorporation of deoxynucleotide triphosphates into the growing strand of nascent DNA. The incorporation of these dNTPs results in the proportional release of pyrophosphate, which is converted into ATP to aid in a subsequent conversion of luciferin to oxyluciferin. The amount of light released in the process is proportional to the number of nucleotides incorporated, and the procedure provides a quantitative portrait of the methylation profile for the amplicon in question.

Key words PCR, Pyrosequencing, Sequencing-by-synthesis, Methylation, Epigenetics

1 Introduction

Pyrosequencing is a sequencing-by-synthesis method used to quantitatively determine the methylation of individual CG cytosines from PCR amplicons produced using a bisulfite-modified DNA template. The pyrosequencing approach involves isolating the template strand from its complementary strand following PCR amplification, accomplished as a result of incorporation of biotin at the 5' end of the primer used to initiate amplification of the particular strand destined to undergo pyrosequencing. Following PCR amplification, the biotin-labeled double-stranded PCR amplicon is bound to streptavidin beads that are then immobilized on vacuum filtration tips that allow for removal of the complementary (non-biotin containing) strand through denaturation and purification. The single-stranded DNA is then annealed to a sequencing primer and undergoes pyrosequencing, allowing for the incorporation of deoxynucleotide triphosphates (dNTPs), one at a time, in a template-dependent, stepwise fashion.

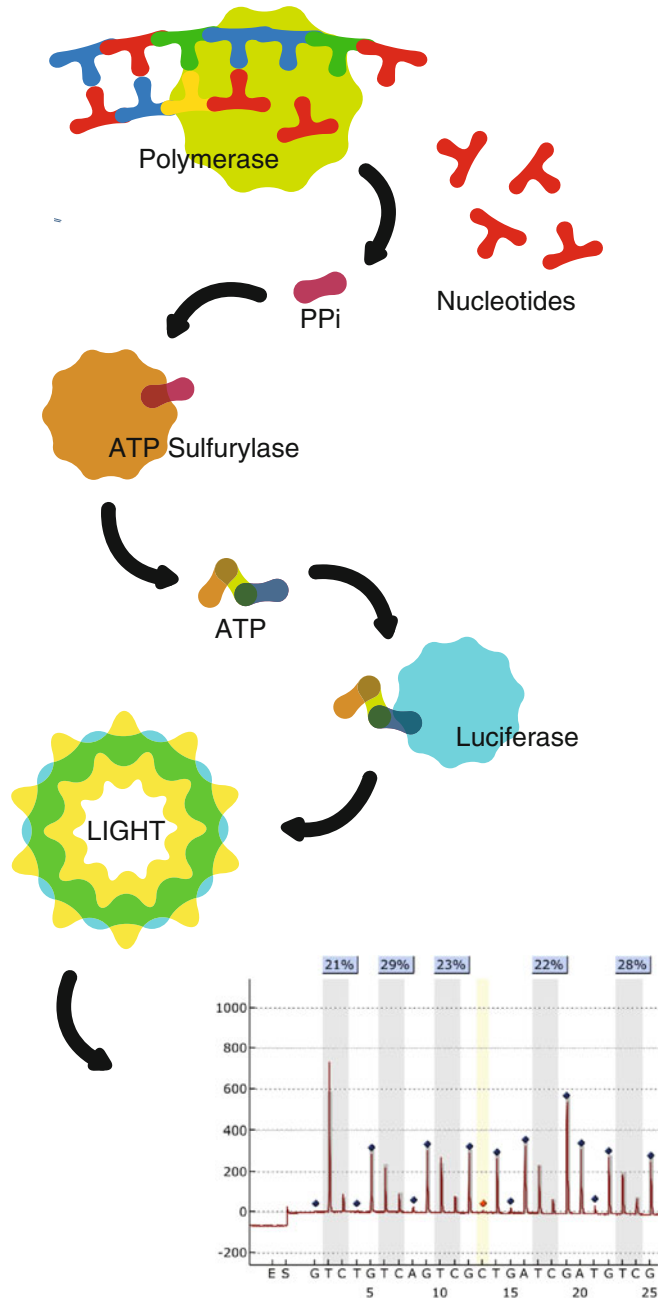


Fig. 1 Bisulfite pyrosequencing. The pyrosequencing reaction uses a single-stranded, biotin-labeled template and involves the addition of a single nucleotide at a time into the reaction mix. Following incorporation of the nucleotide into the nascent complementary strand by DNA polymerase, pyrophosphate is released in a stoichiometric manner and is used to form ATP by ATP sulfurylase. The ATP then fuels production of oxyluciferin from luciferin by luciferase, whereby light is emitted that is proportional to the amount of ATP produced and thus to the number of nucleotides incorporated. The pyrosequencing instrument measures the light produced and plots this on a graph called a pyrogram. Any unincorporated

As each nucleotide is incorporated by the polymerase into the growing strand, pyrophosphate is released. The pyrophosphate is then converted to ATP by ATP sulfurylase and serves as fuel for the luciferase-mediated conversion of luciferin to oxyluciferin, which releases visible light that is proportional to the amount of pyrophosphate initially released and thus to the number of nucleotides incorporated (Fig. 1). Any residual unincorporated nucleotides that remain in the reaction mixture are degraded after each step by apyrase. The intensity of the light emitted for each reaction well is used to both provide information regarding the quantity of template present and also to confirm that the sequence produced matches the sequence of the template. Cytosines that are potentially methylated are treated as variable positions in the sequence, whereby sequential addition of bases corresponding to methylated (C) and unmethylated (T) cytosines provides the means to quantify methylation by measuring peak heights reflecting the incorporation of cytosine relative to the total incorporation for cytosine plus thymine at that particular position. These percentages are automatically calculated by the PyroMark[®] CpG Software for each of the CG cytosines within the sequence analyzed.

Over 84,000 predesigned pyrosequencing assays are available for purchase through Qiagen (PyroMark[®] CpG Assays; www.qiagen.com). Alternatively, pyrosequencing assays can be designed using PyroMark[®] Assay Design Software (Qiagen; Valencia, USA). A thorough description of assay design is beyond the scope of this chapter, but in our experience there are several critical factors to achieve success. The pyrosequencing reaction is carried out at a relatively low temperature and as such is vulnerable to mispriming events from promiscuous priming of the sequencing primer but also potentially from carry forward of any unincorporated biotin-tagged primer or even from template looping. The PyroMark[®] Assay Design Software detects and evaluates these potential mispriming events. This information is used as part of the criteria for determining the suitability of the assay design. Pyrosequencing

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Fig. 1 (continued) nucleotides are degraded by apyrase (not shown) prior to injection of the next nucleotide in to the reaction mix. The y axis of the pyrogram represents the light produced and the x axis provides a readout of the sequence to analyze. Note that multiple sequential same nucleotides in the template will result in incorporation of a proportional amount of the complementary nucleotide at one pyrogram peak position, such that two of the same nucleotides in a row will produce a peak height that is twice as big as that for a single nucleotide incorporation, and so on. The % methylation measured for each CG cytosine is shown above the pyrogram and is calculated by the software. The single nucleotide width vertical shading at nucleotide position 13 indicates a bisulfite control representing a non-CG cytosine in the original sequence. “E” represents injection of enzyme at the beginning of the run, “S” indicates the injection of substrate, both of which should not produce measurable light

using current technology is limited to no more than ~115 bp in length. PCR amplicons for pyrosequencing should be between 150 and 250 bp in length to avoid potential amplification bias and to minimize potential for mispriming [1].

We use Qiagen's Epiect Control DNAs for pyrosequencing assay validation prior to use in evaluating methylation in research specimens by preparing and running replicates of defined mixtures of the fully methylated and unmethylated DNAs as follows: 100%U, 25%M:75%U, 50%M:50%U, 75%M:25%U, and 100%M. This allows us to determine the degree of technical variability, to detect the potential bias in amplification of the methylated versus unmethylated sequence, and to ensure that the assay is able to detect a linear increase in methylation across the dynamic range of the assay.

There are multiple procedural steps required for pyrosequencing, and as such Subheading 3 is broken into subsections that describe the PCR reaction, setting up the pyrosequencing run, procedures for preparing the reagent and capillary tips, preparing the master mix and primer, preparing the nucleotides, preparing the PCR product and adding nucleotides to the tips, performing the dispensation test and preparing the workstation, loading the plate and beginning the run, workstation cleanup, and data analysis and interpretation.

Bisulfite pyrosequencing is particularly useful for not only detailed and accurate quantitative analysis of methylation at specific loci but also analysis of repeat elements, often used as a proxy to assess how methylation is altered throughout the genome. Once assay conditions are optimized, pyrosequencing is amenable to high-throughput analysis of many specimens since the assays are carried out in 48-well or 96-well plate format. We have shown that pyrosequencing can distinguish 5 % differences in methylation using defined mixtures of methylated and unmethylated template DNAs [2]. Pyrosequencing has been used to evaluate DNA methylation profiles in multiple studies of cancer, including gynecologic malignancies [3–6].

2 Materials

2.1 Reagents and Supplies

1. PyroMark® PCR Kit containing 2× PyroMark® PCR Master Mix, 10× CoralLoad™ Concentrate, 25 mM MgCl₂ stock, and Nuclease-free water.
2. PCR Primers (one with 5' biotin label, HPLC purified; *see* Subsection 3.1, PCR, below).
3. 96-well plate.
4. Nuclease-free H₂O.
5. PyroMark® Binding Buffer.
6. PyroMark® Annealing Buffer.

7. GE Healthcare Streptavidin Sepharose™ High-Performance Beads.
8. Pyrosequencing Primer.
9. PyroMark® Gold Q96 CDT Reagents including PyroMark® Enzyme Mixture, PyroMark® Substrate Mixture, and PyroMark® dNTPs (dATP α s, dCTP, dGTP, and dTTP).
10. Plate Sealing Film for Raised Edge Plates (Denville Scientific, Inc.).
11. PyroMark® Wash Buffer, 10 \times .
12. PyroMark® Denaturation Solution.
13. PyroMark® Q96 HS Plate.
14. PyroMark® Q96 HS Capillary Tips (CDTs).
15. PyroMark® Q96 HS Reagent Tips (RDTs).
16. Aluminum Plate Seals (Greiner Bio-One SILVERseal).

2.2 Equipment

1. PyroMark® Q96 MD Pyrosequencing Instrument.
2. PyroMark® Q96 Vacuum Workstation.
3. 96-well Capacity Thermocycler.
4. Eppendorf MixMate plate mixer (*see Note 1*).
5. Heat block, prewarmed to 85 °C.

3 Methods

3.1 PCR

PCR for pyrosequencing requires use of a forward and reverse primer, one of which is modified by the addition of a biotin molecule at the 5' end. The primer containing the biotin molecule should be the one used to synthesize the strand that will be used as template for pyrosequencing. Without the biotin, the amplified DNA will not be retained on the streptavidin beads for analysis in later steps and will instead pass through the filter probes of the pyrosequencing workstation. Optimization of PCR conditions for the primers is required prior to their use in pyrosequencing. The goal of the optimization is to produce a single, robust band of the correct size as detected by ethidium bromide staining following agarose gene electrophoresis. Due to the added expense of the biotin modification of the primer, we typically order standard primers for use during the PCR optimization process. Once the optimal PCR conditions are defined, we order the appropriate primer with the 5' biotin modification and with HPLC purification.

1. Prepare the PCR Master Mix in a nuclease-free tube to bring the volume per reaction to 25 μ L after the addition of bisulfite modified DNA in **step 2** (*see Table 1 and Note 2*).

Table 1
PCR Master Mix

Stock	Reaction mix volumes (μL)	Final concentration
Reverse primer	0.3 μL	120 nM
Forward primer	0.3 μL	120 nM
25 mM MgCl_2	1.5 μL	1.5 μM
10 \times Coral Load™ Concentrate	2.5 μL	1 \times
2 \times PyroMark® PCR Master Mix	12.5 μL	1 \times
DNA (to be add later)	X	20–50 ng/reaction
Nuclease-free water	X	
Total volume	25 μL	

2. Add bisulfite modified DNA (20–50 ng) to the PCR master mixture in each reaction well. After sealing the tube, vortex, and quick spin to collect contents at the bottom of tubes.
3. Transfer the reaction wells to a thermocycler and perform PCR using the conditions most appropriate for an analysis of the gene in question.
4. After the PCR has finished, the sample may either be removed from the thermocycler for immediate analysis or stored at $-20\text{ }^\circ\text{C}$.

3.2 Pyrosequencing: Setting Up the Run

1. Open PyroMark® CpG Software 1.0.11.
2. For a new assay, open a new file in Pyro Q-CpG design folder, right click and choose “New Assay.” Copy/paste the sequence into the “sequence to analyze” window and click on “generate dispensation order.” Add bisulfite control sites at the orange “T” sites as designated by the software, which are preselected as bisulfite controls and represent non-CG cytosines in the original sequence. The CG sites are highlighted in gray and the BS control sites are in orange.
3. Select “File \rightarrow New Run” to access the run setup menu. The run setup menu can also be accessed by clicking on the “New Run” icon located beneath the toolbar.
4. There will be three smaller run setup windows that appear on the right of the screen. The upper left screen shows the run name, instrument parameters, plate ID, reagent ID and also has a small window available for entering notes.
5. Enter run name and plate ID in the run setup menu.
6. Select “Instrument Parameters” according to the CDT version used. The parameter information for specific CDT versions

can be found at www.qiagen.com and is based on the lot number of the CDTs purchased.

7. The lower panel on the right-hand side of the screen shows the plate wells. There are three text boxes for each well. The uppermost text box is used to designate which assay you want to run, selected from the pre-entered assay designs. Select the appropriate assay in the Pyro Q-CpG design folder and drag and drop this on the “Assay Name” in the top text box of the first well. Click the box and drag the cursor across all other wells to assign that same assay information via auto-fill, as appropriate.
8. Enter the sample ID into the middle textbox of each well in the “Plate Wells” panel. The lower text box can be used for adding any desired notes.
9. After all of the above information has been entered, the right upper panel will show the well information with the assay design, sample ID, and notes. Double check to ensure that all information is correct.
10. For reagent and capillary tip volume information, select “Tools → Volume Information.” Enzyme and substrate volumes are listed under “E-mix” and “S-mix”, respectively. Nucleotide volumes for capillary tips are listed according to each base. Note these volumes for a later step in the protocol.

3.3 Cleaning Reagent and Capillary Tips

Cleaning of the reagent and capillary tips should be performed both prior to and after a run (*see Note 3*).

1. To wash a reagent (RDT) or capillary (CDT) tip, first rinse the interior of the tip with nuclease-free water five times by filling to the brim with nuclease-free water and inverting to empty.
2. After the tip has been rinsed, finish cleaning by “milking” five times. To milk the tip, first fill to the brim with nuclease-free water and then create a seal at the top by completely covering the opening with your thumb. Then, lightly apply pressure to the top and sides of the tip using the thumb and forefingers of the opposite hand, respectively, until a straight stream of water can be steadily produced.
3. Once tips have been rinsed and milked prior to use in the pyrosequencing run, place each tip upside down on a Kim wipe until use.

3.4 Preparing the PCR Components

1. To prepare the master mix, in a 15-mL conical tube combine 2 μL of Sepharose streptavidin-coated beads, 40 μL of PyroMark® Binding Buffer, and nuclease-free water to bring the volume to 80 μL for each of the reactions after accounting for addition of the PCR product (plus some additional to allow for pipetting error) (*see Note 4*).
2. Invert gently several times to mix (do not vortex).

3. Immediately (before the beads settle) pipet the appropriate amount of master mix into each well of a PCR plate for each sample to be analyzed (80 μL minus the amount of PCR product to be added). Place on ice.
4. To dilute the primer for an entire 96-well plate, add 4 μL of appropriate sequencing primer (100 μM) to 1,196 μL of PyroMark[®] Annealing Buffer and vortex thoroughly. Place on ice.
5. In four separate tubes, prepare each of the four PyroMark[®] dNTPs by mixing each dNTP with an equal volume of TE Buffer to make a final volume given by “Tools \rightarrow Volume Information” (*see* **step 10** from Subheading 3.2).
6. Add PCR product (8–10 μL) to the master mix in each well (from **step 3**, Subheading 3.4).
7. Seal the plate thoroughly using an aluminum plate seal.
8. Place the plate on the plate mixer at 1,400 rpm for 7–10 min at room temperature.
9. Load each dNTP mixture into the corresponding CDT in alphabetical order (A, C, G, T). Load the required volumes of enzyme and substrate into the RDT tips. Place each CDT and RDT tip into its corresponding slot in the cartridge (*see* **Note 4**).

3.5 Dispensation Test and Preparing the Pyrosequencing Reactions

1. To insure proper functioning of the tips, a dispensation test, controlled by the software, is performed prior to the actual run whereby reagents and nucleotides are dispensed by the pyrosequencer onto a clear plastic plate cover and should form droplets that are easily visible and of uniform size. Open the outer lid of the PyroMark[®] Q96 MD. Load the reagent cartridge into the instrument and select “Open Process Chamber” from the software menu. Once the process chamber is open, load a dispensation test plate with a clear plastic cover on top and click on “Close Process Chamber.” Then, click on “Test Dispensing Tips” to begin the dispensation test.
2. To prepare the workstation, load troughs 1–4 with 110 mL 70 % EtOH, 90 mL PyroMark[®] Denaturation Solution, 110 mL nuclease-free water, and 110 mL PyroMark[®] Wash Buffer, respectively. Load the “Parking” trough with 180 mL nuclease-free water and submerge the filter tips of the vacuum prep tool in the nuclease-free water.
3. Return to the computer, close the “Test Dispensing Tips Finished” message, and open the process chamber lid. The dispensations should appear as six rounded droplets. If any of the droplets are not present or appear aberrant, repeat the dispensation test. If the problem persists after multiple dispensation tests, rinse tips and test for flow-through of liquid using the manual cleaning procedure described above in “Cleaning Reagent and Capillary Tips.”

4. Vortex the diluted primer and add 12 μ L to each well in a PyroMark[®] Q96 plate. Once the primer has been plated, slide the PyroMark[®] Q96 plate into the PyroMark[®] plate platform in the vacuum workstation.
5. Remove the plate with the PCR product from the vortexer and peel off the aluminum seal. DO NOT quick spin the PCR product plate at this point. Then, check to make sure that the vacuum seal on the liquid waste container at the workstation is tightly sealed.
6. Remove the prep tool from the parking trough and start the pump. Apply a vacuum to the prep tool by turning the workstation to ON. Keep the vacuum ON THROUGH **step 9** so that the beads remain attached to the filter probes. Matching position A1 of the prep tool to position A1 of the PCR product-beads plate, slowly lower the filter probes into the plate wells until the entire PCR product mixture has been aspirated through the filter tips and the streptavidin beads have been immobilized on the probes. Then, gently remove the prep tool from the plate, taking care not to dislodge the streptavidin beads from the filter probes. Aspiration of the liquid and immobilization of the beads on the filter probes should take roughly 15 s.
7. While maintaining the vacuum, place the prep tool filter probes directly into the 110 ml of 70 % EtOH in trough 1. Once fluid is visible in the tubing, continue aspiration for 5 s, then remove the prep tool from the 70 % EtOH while maintaining its orientation and allow the remaining 70 % EtOH to be fully aspirated from the prep tool by the vacuum.
8. Place the prep tool into the 90 mL of PyroMark[®] Denaturation Solution in trough 2. Once fluid is visible in the tubing, continue aspiration for 5 s, remove the prep tool and allow the Denaturation Solution to be fully aspirated from the prep tool by the vacuum.
9. Place the prep tool into the 110 mL of PyroMark[®] Wash Buffer in trough 3 and aspirate the Wash Buffer completely. Lift the prep tool out of the Wash Buffer and, while maintaining its orientation, turn the workstation to OFF, stop the pump, and release the vacuum on the liquid waste container by loosening the lid.
10. Lower the prep tool into the primer-loaded PyroMark[®] Q96 plate in the plate platform of the workstation, so that the filter probes rest in their corresponding wells (A1 to A1). Gently rock the prep tool from side to side to release the beads into the wells.
11. Place the PyroMark[®] Q96 plate on a prewarmed PyroMark[®] Q96 Sample Prep Thermoplate. There are two parts to the Thermoplate; the PyroMark[®] Q96 plate should be sandwiched

between the upper and lower blocks and should be fully covered by the top block. Place the PyroMark® Q96 plate-Thermoplate onto the surface of the heating block at 85 °C for 2 min.

12. After 2 min, switch the heating block off but do not remove the PyroMark® Q96 plate-Thermoplate from the heating block. Allow the PyroMark® Q96 plate-Thermoplate to sit atop the (now cooling) heating block for another 2 min. Then, remove the PyroMark® Q96 plate from between the two blocks of the Thermoplate and let cool to room temperature (~5 min). Once the PyroMark® Q96 plate has reached room temperature, move on to the next part of the protocol.

3.6 Loading the Plate and Beginning the Run

1. Click on “Open Process Chamber” and insert the PyroMark® Q96 plate into the PyroMark® Q96 MD instrument, and select “Close Process Chamber.”
2. Begin the run by clicking the “Start Run” arrow button located next to the run parameters.

3.7 Workstation Cleanup

1. Place the filter probes of the prep tool into the 110 mL of nuclease-free water in trough 4 and gently agitate for 10 s.
2. Tighten the vacuum lid on the waste container, start the pump, and turn the workstation to ON to flush the filter probes for ~5 s.
3. Lift the prep tool from the water, and aspirate the water through the prep tool.
4. Stop the pump, turn the workstation to OFF, release the vacuum and return the prep tool into a clean storage box until next use (*see Note 5*).

3.8 Data Analysis and Interpretation

1. When the run is complete, the data for each sample will be analyzed by the software and scored as “passed,” “check quality,” or “fail.”
2. Run data for each specimen should be inspected to insure that adequate peak heights were achieved (~150–300 light units for a single-nucleotide incorporation). Low peak heights are more likely to reflect stochastic results from having too little template or loss of beads during the wash steps on the workstation, even if the run was scored as “passed.”
3. The pyrogram software output produced for each sample provides a linear representation of the sequence read based on the intensity of light produced after each incorporation. Peak heights will proportionately increase with the number of same sequential nucleotides in the template being sequenced. For example, the template sequence 3'-A-G-G-T-T-T-A-T-5' will show results in which the first incorporation will be a single

peak height T, the second will be a double peak height C, the third will be a triple peak height A, the fourth will be a single peak height T, and the fifth will be a single peak height A. Deviations in the expected peak heights, based on those anticipated from the input sequence, will trigger the software to score the run as a “check quality” run or a “fail.”

4. The pyrosequencing software treats cytosines within “CG” dinucleotide positions within the sequence much like a polymorphic site, since either a C or T is possible at the cytosine position depending on if this particular cytosine was methylated or unmethylated, respectively, in the original sequence. Thus, the sequence to analyze will contain a “T” adjacent to a “C”. The light produced from each of these peaks is used to determine the proportion of cytosine that is methylated in the sample at that position by calculating the light intensity produced from incorporation of the C relative to the total produced from combined C + T incorporation. The percent methylation calculated is provided as a visual readout above each CG position within the sequence in the pyrogram.
5. Bisulfite controls are also a necessary component of pyrosequencing and are automatically chosen and inserted into the sequence to analyze by the PyroMark® CpG Software. These controls represent cytosines in the original sequence that are not in CG context and therefore should be fully converted by the bisulfite treatment to uracils (thymine following PCR). A “T” adjacent to a “C” is also used for these positions, just as for CG cytosines (described above), except in this case the results should show near complete incorporation for the “T”. The proportion of “C” incorporation at these positions is indicative of the degree to which conversion was incomplete, at least at that position. This is calculated by the software, which has a default threshold setting of 96.5 % conversion (4.5 % incomplete conversion of cytosines) before a sample will fail the run. This threshold setting can be adjusted using the software settings as desired. It is important to note that incomplete conversion will falsely elevate the signals coming from CG positions. In our hands, conversion efficiencies are typically >98 % for high quality, high purity bisulfite modified genomic DNA.

4 Notes

1. It is important to use a mixer that can thoroughly mix the reagents in a 96-well plate while not causing the reagents to spill or splash up onto the sides of the tubes or the plate seal. We have tried several brands and the Eppendorf MixMate works well in our hands.

2. It is strongly recommended to include controls on each plate for both the PCR reactions (e.g., positive control, no template control, etc.) and pyrosequencing reactions (annealing buffer plus template but without primer, primer plus annealing buffer but without template) along with positive and negative controls for methylation status. We use the EpiTect Control DNAs from Qiagen that are provided in bisulfite modified form. These are produced using whole genome amplification followed by treatment with or without the bacterial *SssI* methyltransferase. *SssI* methyltransferase enzymatically methylates all CG cytosines throughout the genome to generate the fully methylated DNA while the methylation pattern in the original template is effectively “erased” by the whole genome amplification to produce the unmethylated DNA. These controls are especially useful for assessing assay performance for regions of the genome that normally exhibit endogenous methylation, such as imprinted loci, since it is extremely difficult to obtain naturally occurring DNA specimens that exhibit a complete lack of methylation at these loci for use as unmethylated controls.
3. It is critical to clean the tips well, as unexpected peaks might otherwise be generated in the subsequent sequencing run. These unexpected peaks can be caused by pyrophosphate generated as a result of reagent(s) remaining in unclean tips. We have found that with appropriate handling, CDTs can be cleaned and reused for up to 15 runs and RDTs for up to 8 runs.
4. Due to light sensitivity, minimize exposure of the reagents to light.
5. An empty pipet-tip box can be used as a storage box such that the filter probes are gently inserted into the empty holes and thus kept clean and protected from damage.

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