Chapter 8

Pyrosequencing Analysis for Breast Cancer DNA Methylome

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Abstract

Unraveling DNA methylation profile of tumor is important for the diagnosis and treatment of cancer patients. Because of the heterogeneity of clinical samples, it is very difficult to get methylation profile of only tumor cells. Laser capture Microdissection (LCM) is giving us a chance to isolate the DNA only from the tumor cells without any stroma cell's DNA contamination. Once we capture the breast tumor cells, we can isolate the genomic DNA which is followed by the bisulfite treatment in which unmethylated cytosines of the CG pairs are converted into uracil; however, methylated cytosine does not go into any chemical change during this reaction. Next, bisulfite treated DNA is used in the regular PCR reaction to get a single band PCR amplicon which will be used as a template for the pyrosequencing. Pyrosequencing is a powerful method to make a quantitative methylation analysis for each specific CG pair.

Key words Breast cancer, Laser capture microdissection (LCM), DNA methylation, Bisulfite conversion, Pyrosequencing

1 Introduction

Breast cancer is the most common form of cancer among women and it is the second most deadly cancer after lung cancer [1]. Since tissue samples taken from cancer patients are heterogeneous, and therefore are composed of a mixture of tumor cells and surrounding stroma cells, experimental techniques for the analysis of cancer cells have been difficult. However, improvements have been made over the years, including Laser Capture Microdissection (LCM), which has been used for the analysis of histopathology samples since 1996 [2]. In this technique, laser is accompanied with the objective of the microscope and focuses on the tissue sections. One can draw a border around target cells on the computer image of histology slides which is called "element" to dissect the cells in the small size of tube which will be used for future DNA or RNA isolation. Therefore, LCM improves the quality of data from clinical samples and gives us a trustable output [3].

Jian Cao (ed.), *Breast Cancer: Methods and Protocols*, Methods in Molecular Biology, vol. 1406,
DOI 10.1007/978-1-4939-3444-7_8,

Along with genetic alterations of individuals, recent findings shed a light on the importance of epigenetic aberrations for cancer progression [4, 5]. Epigenetic mechanisms are basically divided into two main groups called DNA methylation and histone modifications. Some studies include miRNA as part of the epigenetic mechanism. The methylation of the fifth carbon on the cytosine (5mC) has been known as the main mechanism of DNA methylation for gene regulation in eukaryotes, but very recent studies demonstrated that sixth nitrogen on the adenosine (6mA) can also be methylated and change the gene expression in eukaryotes as well [6, 7]. For the rest of the chapter, the term of DNA methylation is used only for the methylated cytosine (5mC). In normal cells, most of the DNA methylation takes place in repetitive genomic regions, such as LINES (long interspersed transposable elements) and SINES (short interspersed transposable elements) to maintain genomic integrity [8]. Besides random and diverse methylation of cytosines in these regions, DNA methylation is usually concentrated in CpG islands (CGI), which are described with the following formula; minimum 200-bp stretch of DNA with a minimum C + G content of 50 % and an Obs_{CpG}/Exp_{CpG} in excess of 0.6 [9]. Based on this criterion, there are approximately 29,000 CpG islands (CGI) in the human genome. Besides the localization within promoters of genes, some CpG islands exist in the intergenic or intragenic (intron) regions [10]. Half of all human promoters have been reported to have CGIs, but these CGIs within promoters are generally not methylated. Increased level of methylation in the CpG island around promoters inhibits transcription either directly by blocking the access of specific transcription factors to the promoter of the genes or indirectly by recruiting methyl-CpG-binding domain (MBD) proteins which might recruit histone-modifying and chromatin-remodeling complexes that cause the condensation of the chromatin [11]. In addition to the basic role of DNA methylation in the development of organisms and cellular differentiation via altering gene expression profiles, aberrant DNA methylation patterns have also been linked to nearly all types of cancer [12]. The role of methylation in cancer progression is primarily considered as a molecular instrument for hypermethylation of promoters in order to silence tumor suppressor genes, and majority of studies related with this topic focus in this direction [13, 14]. On the other hand, hypomethylation of oncogenes or growth-related genes is another mechanism related with cancer progression. Almost 30 years ago, Feinberg and Vogelstein demonstrated for the first time that hypomethylation in the promoters of oncogenes, such as c-Ha-ras and c-Ki-ras, induced the formation of tumors in colon and lung tissues [15]. Therefore, monitoring the alteration of DNA methylation level on specific loci, especially the promoter region of tumor suppressor and oncogenes, is very critical for cancer patients. Frommer and his

colleagues developed one of the most powerful techniques in which unmethylated cytosines, not methylated ones, are converted into uracil after bisulfite treatment. Finally, we can identify the status of the cytosine after the Sanger sequencing of the target region [16, 17].

In 1996, another sequencing technique called pyrosequencing was developed by Mostafa Ronaghi and Pal Nyrén [18, 19]. In their new sequencing approach, they immobilize one strand of DNA with the help of biotinylated primer and then they add the pyrosequencing primers into the solution. Later, they add and remove A, G, C, and T solution for each nucleotide synthesis. In each step, pyrophosphate (PPi) is released because of the addition of one of dNTPs to the growing strand with the help of DNA polymerase. ATP sulfurylase catalyzes the reaction between this free PPi and APS (adenosine 5′ phosphosulfate) after each step to produce an ATP which is used as a substrate in the next step. Luciferin is converted into oxyluciferin in the presence of ATP, producing light. Then all free single nucleotides and ATP are degraded by another enzyme called apyrase. Amount of light produced at each step is used to quantify the data and predict the percentage of nucleotide for some clinical analysis. Main applications of pyrosequencing are quantitative analysis of sequence variants for allele frequency in the population, analysis of mutations, bacterial/fungal typing, and analysis of DNA methylation ratio on specific CG pairs. The main disadvantage of pyrosequencing is the short read outcome that is usually around 50–60 bp.

2 Materials

2.1 Cell Cultures or Tissues

1. Breast cancer MCF-7 and MDA-MB-231 cell lines were purchased from ATCC (Manassas, VA) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) plus 10 % FBS. Breast cancer invasive and in situ samples were obtained from the Pathology Department of Stony Brook University.

2.2 FFPE Tissue Preparation

1. Breast cancer specimen (right after surgery, standard sample size: $0.5 \times 1 \times 1$ cm).
2. 10 % neutral formalin.
3. Histology cassette (Tissue-Tek® Uni-Cassettes, Sakura).
4. 70, 80, and 95 % EtOH.
5. Xylene.
6. Paraffin.
7. Hot water bath (60 °C).
8. Cooling station (<−10 °C).

2.3 Hematoxylin and Eosin Staining

1. Microtome (Model 48577-60).
2. SuperFrost Plus slides.
3. 50 °C water bath.
4. 65 °C incubator.
5. Xylene
6. 70, 80, 95, and 100 % EtOH.
7. Hematoxylin solution (*see* **Note 1**).
8. Acid alcohol: 0.1 % HCl, 50 % EtOH in distilled water.
9. Scott's tap water: 2 g sodium bicarbonate and 10 g $MgSO_4$ in 1 l of distilled water.
10. Eosin solution.
11. Glass or plastic jars.
12. Mounting media.
13. Forceps and brushes.

2.4 Laser Capture Microdissection

1. Leica laser microscope.
2. PALM RoboSoftware.
3. 0.5 ml eppendorf tube.
4. 70 % EtOH.
5. Microcentrifuge.

2.5 Genomic DNA Isolation

1. Qiagen DNeasy Tissue Kit (Cat # 69504).
2. Tabletop centrifuge (up to 13 k rpm).
3. Xylene.
4. RNase.

2.6 Identification of CpG Island

1. MethPrimer: http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi.
2. Bioinformatics.org: http://www.bioinformatics.org/sms2/cpg_islands.html.
3. CpG Island Searcher: http://www.uscnorris.com/cpgislands2/cpg.aspx.
4. UCSC Genome Bioinformatics: https://genome.ucsc.edu/index.html.

2.7 Primer Design and CpG Assay Design

1. MethPrimer [20]: http://www.urogene.org/methprimer/index.html.
2. MethMarker: http://methmarker.mpi-inf.mpg.de/.
3. RepeatMasker: http://www.repeatmasker.org/.
4. Pyro Q CpG (Biotage) and PSQ.

2.8 Bisulfite Treatment and Bisulfite-Specific PCR

1. EZ DNA Methylation-Gold™ Kit (Zymo Research).
2. PCR machine.
3. High fidelity DNA polymerase or HotStarTaq DNA Polymerase (store at –20 °C).
4. 5′-biotinylated and HPLC purified forward primers (10 nM, Sigma or Eurofins).
5. Unlabelled reverse PCR primer (desalted, 10 nM).
6. QIAquick PCR Purification Kit (Qiagen, Cat. No. 28104).
7. Human non-methylated control DNA (D5014-1, Zymo Research).
8. HpaII restriction endonuclease (NEB).

2.9 Gel Electrophoresis

1. Agarose.
2. Gel electrophoresis system.
3. TAE buffer (50× stock) : 242 g Tris base, 57.1 ml glacial acetic acid, and 100 ml of 500 mM EDTA (pH 8.0) solution in 1 l of distilled water.
4. 6× loading dye: 3 ml glycerol (30 %), 25 mg bromophenol blue (0.25 %) in 10 ml distilled water.
5. UV gel analyzer.
6. MinElute Gel Extraction Kit (if necessary) (Qiagen).

2.10 Preparation of Pyrosequencing Template

1. Heat block up to 80 °C.
2. Microplate shaker.
3. Troughs and 96-well vacuum prep.
4. 96-well pyrosequencing plate.
5. Streptavidin Sepharose High Performance Beads (keep at 4 °C).
6. Annealing buffer: 2.42 g Tris and 0.43 g magnesium acetate-tetrahydrate in 1 l distilled water (pH: 7.6).
7. 2× Binding buffer: 1.21 g Tris, 117 g NaCl, 0.292 g EDTA, and 1 ml Tween 20 in 1 l distilled water (pH:7.6).
8. 70 % EtOH.
9. Denaturation solution: 0.2 M NaOH.
10. Washing buffer: 1.21 g Tris in 1 l distilled water (pH: 7.6).
11. Unlabeled internal pyrosequencing primer.
12. Adhesive sealing film for 96-well plate.

2.11 Pyrosequencing

1. Pyromark Q24 or equivalent instrument.
2. Pyromark Q24 or equivalent cartridge.

2.12 Run Sample and Data Analysis

Pyro Q CpG Software.

3 Method

3.1 Cell Cultures and Tissues

Cell lines or tissues both can be used for the pyrosequencing analysis of DNA methylome. For cell lines, adherent cells need to be harvested with trypsin (0.05 %) for 2–5 min at 37 °C incubation. Then, cells will be washed with PBS and pellet can be used for the DNA isolation in Subheading 3.5. If you have paraffin embedded tissue, you can continue to Subheading 3.3 otherwise breast tissues should be prepared immediately after the surgery under the supervision of pathologist.

3.2 FFPE Tissue Preparation

1. Dissect the breast tumors with these dimensions; 0.5 cm (depth) × 1 cm × 1 cm and place inside a histology cassette between two small sponges. Put the cassette immediately to 10 % formalin solution and incubate for 1–2 days at room temperature (*see* **Note 2**).
2. Use tap water to wash the fixed tissue for half hour. Dehydrate the tissues by 30 min incubation of 70, 80, 95 % of EtOH, followed by two times of 100 % EtOH incubation, 1 h each.
3. Wash the tissue with xylene twice, 1 h each. At the same time, start to prepare paraffin by heating it to 55–60 °C.
4. Pour a little hot paraffin into the mold, then embed the fixed tissue on top of hot paraffin. Orient the specimen if it is necessary, otherwise fill the plastic cover with hot paraffin. Once mold is filled with hot paraffin, move the mold onto the cooling station (<–10 °C).
5. Take the paraffin block from mold once the wax is hard.

3.3 Hematoxylin and Eosin Staining

1. Set up water bath to the 50 °C. Change the blade of microtome with a fresh one and set up the thickness to 5 μm. FFPE sections were cut and collected from block face and transferred onto a prewarmed water bath with the help of brush (*see* **Notes 3** and **4**). Let paraffin sections incubate on the surface of warm water for 5 min, then you can transfer tissue sections on the surface of SuperFrost Plus slides gently. Mark your samples with pencil; do not use pen (*see* **Note 5**).
2. Transfer all your slides to the 65 °C incubator or small oven for 2 h. This treatment melts most of the paraffin around your tissues.
3. After drying the sections on a glass slide, the sections are merged into xylene solution for 10 min, twice. The sections then are rehydrated in serial ethanol solution from 100 % EtOH to distilled water in the following order: 100, 95, 70, 50 % EtOH and water, 10 min each.
4. The slides are stained with a nuclear dye (Hematoxylin Gill-1 or Mayer solution) for 5 min followed by rinsing with distilled

water. Then, the slides are incubated in acid alcohol solution for 3 s. After washing the slides in tap water, they are treated with eosin (counter stain) for 30 s to 1 min.

5. The slides then are dehydrated in several ethanol solutions in the following order: 70, 95, and 100 % EtOH, 10 min each. Finally, the slides are treated with xylene for 5 min, twice. The first section of every six cut should be covered with coverslips and used as a reference slide for the others.
6. The slides can be stored at room temperature.

3.4 Laser Capture Microdissection

1. First tumor cells should be identified on the reference slides for each H and E (hematoxylin–eosin)-stained sections. In this step, it is recommended that you should get help from a pathologist if you do not have enough experience to differentiate the tumor cells (in situ or invasive) from surrounding stroma cells.
2. By using the reference slide you can identify the location of tumor cells and mark their location on the bottom of the slide with a pen. Then, place the marked slide on the microscope stage with the help of slide clips. Please see the summary of the setup in Fig. 1.
3. Put 40 μl of 70 % EtOH to the 0.5 ml eppendorf tube's cap. Invert the tube carefully and place it on the tube holder of the microscope on top of the slide. Distance between the slide and the cap should be minimized (*see* **Note 6**).

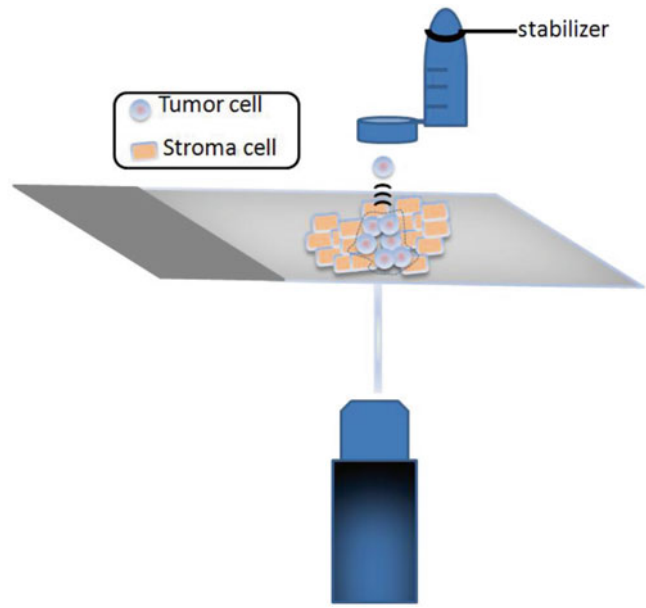

Fig. 1 Positions of the tissue slide, collection tube, and objectives in the microscope

4. Open the PALM ROBO or equivalent application on the computer. First, set up 10× objective on the microscope and observe the image on the screen. By taking advantage of previous mark on the bottom of the slide, move the stage on that position. Optimize the "focus" and "light" on the program and try to see image on the computer. The image quality on the computer might be very low since there is no mounting media and coverslip on the slide. If you see very blurry image, change the objective into 5× and adjust the distance between the slide and eppendorf's cab.
5. Draw a line around the tumor cells on the computer image to catapult the individual points out of your sample. These defined areas are called "elements". You can use graphics bar of PALM RoboSoftware to define different sizes and shapes of elements. You can save your elements into your computer and calculate the catapulted area for your quantitative analysis if you need it (*see* **Note 7**).
6. Set up the speed of the laser to 2000 μm/s for 5 μm section. If it is larger than 5 μm, reduce the speed. Two other parameters need to be adjusted are "UV-energy" and "UV-focus". Initially set up the "UV-energy" to 82 and the "UV-focus" to 76. After the first couple of shots, you need to adjust these values according to the softness of the tissue. If cells are not dissected from the surface, you can increase the energy and focus. The specimens were then subjected to the dissection by hitting the laser in the designated area on the glass slide and cells were collected in the inverted microcentrifuge cap. At the end of dissection, 200 μl of ethanol was added into the microcentrifuge tube. Tubes were centrifuged at 13,000 rpm (17,000–18,000 × *g*) for 15 min. After centrifugation, most of the ethanol was removed from tube and cells were used to extract the DNA (*see* **Note 8**).

3.5 Genomic DNA Isolation

1. Use the whole pellet obtained from **step 6** of Subheading 3.4 for DNA isolation. Perform the DNA isolation according to the manufacturer's protocol (Qiagen DNeasy Tissue Kit). Since you have already treated the tissue with xylene at the end of H and E staining, it is not necessary to treat the pellet with xylene. If your pellet size is large and if it seems to contain wax, treat your pellet with xylene according to the manufacturer's protocol. Since total volume of DNA solution for the bisulfite treatment is around 20 μl, elute your DNA in 20–30 μl of water rather than 100 μl. Or elute the DNA in 100 μl water and concentrate the DNA with vacuum dryer, if the latter is available.
2. Measure the concentration of your DNA with NanoDrop, and aliquot 200–500 ng DNA for the next step. If you have less than 200 ng DNA, continue with your DNA solution.

However, if the total amount of your DNA is less than 500 pg, you need to prepare more slides and perform more laser capturing.

3.6 Identification of CpG Island

1. Based on the bioinformatics tool mentioned in the Subheading 2.6, find your potential CpG island around your target genes. By blatting this region in UCSC genome browser, you can confirm the presence of the CpG island and download the CpG island with 100 bp flanking regions that can be used for the primer design in the next step.

3.7 Primer Design and CpG Assay Design

1. Primer design for bisulfite treated DNA is the critical step for the DNA methylation experiment since all cytosine in the genome except the cytosine of CpG pairs converted into uracil, however the status of cytosine in the CpG pairs after the bisulfite treatment depends on the methylation profile. Therefore, bisulfite sequencing primers should align to the CpG free region. Therefore, it is useful to include some flanking region around your CpG island before starting primer design.
2. By using the "repetitive masker" from Subheading 2.7, you can eliminate repetitive region for your primer design.
3. Some important criteria for pyrosequencing PCR primers;
   (a) They should not form dimers or hairpin structures.
   (b) Length of primers should be between 20 and 30 bp with an annealing temperature of 50–60 °C.
   (c) Product size should be between 100 and 300 bp, 200 bp is the optimum length (It is very difficult to find CpG-free regions for some parts of genome in which CpG intensity is very high; you might increase the size of your PCR fragment up to 500 bp in these cases).
   (d) One of the forward or reverse primers should be biotinylated at their 5′ end.
   (e) There is no large "T" stretches in the target sequence.
4. Biotage PSQ program is one of the programs that can help you to design the bisulfite-specific PCR primers and sequencing primers. First you need to convert all "CG" pairs into "YG" pairs in your target sequence, and then convert all "C" into "T" (Fig. 2). Import this modified DNA sequence into sequence editor. On the sequence tab, you can set your region where you want to design your PCR primer and sequence primer. Hit run button to start analysis. The color of the primer on the right tab demonstrates the quality of the primer sets; Blue primers are of high quality, yellow primers are of medium quality, orange primers are of low quality, and red primers are

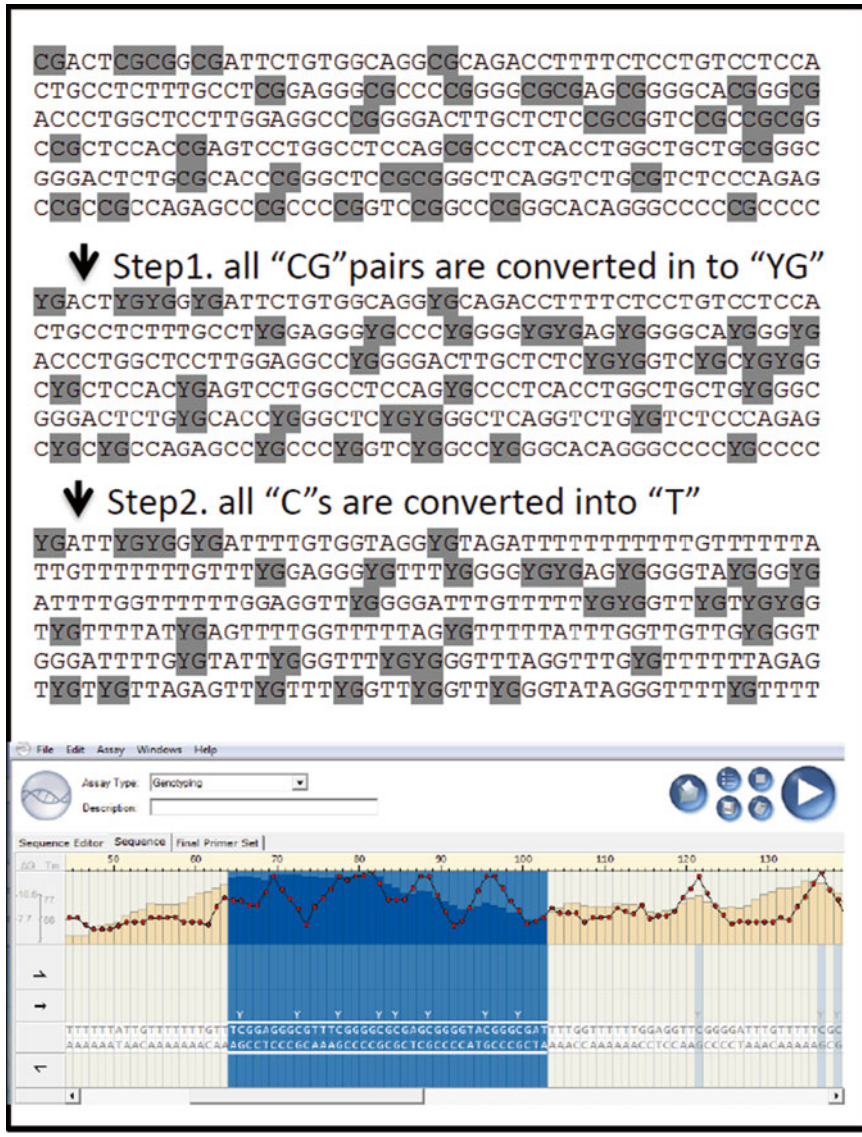

Fig. 2 Preparation of your target sequence for primer design. *Top panel*: original sequence of your target. *Middle panel*: All "CG" pairs converted into "YG" and *shaded with gray*. *Bottom panel*: All other remaining C's in the original sequence are converted into T's. Lowest section demonstrates that how the modified sequence looks like once you import your modified sequence into the Pyro Q CpG program. *Blue shaded area* was chosen for the first assay

of very low quality primer with some severe problem. Usually scores for the primers are in the range of 50–70 out of 100. Warnings for the primers should be taken into consideration before ordering, and they are sometimes eliminated by changing the target site or with a few base pair changes in the position of the primers. You can order blue or yellow primers for your assay and do not forget to add biotin to one of the forward or

reverse primers. If your amplicon size is larger than 100 bp, you can design several sequencing primers since each sequencing primer only reads up to 50 bp in the pyrosequencing run.

3.8 Bisulfite Treatment and Bisulfite-Specific PCR

1. Treat up to 500 ng isolated DNA from the dissected cells and unmetylated control DNA by using EZ DNA methylation Gold kit (Zymo Research) according to the manufacturer's protocol. Suggested treatment on the thermal cycler is:

   98 °C for 10 min, 64 °C for 2.5 h.

   Alternative incubation for bisulfate conversion:

   98 °C for 10 min, 53 °C for 30 min, (53 °C for 6 min + 37 °C for 30 min) for eight cycles,
2. DNA can be eluted in 20 μl water or elution buffer and 2 μl of the elute should be used in the BSP PCR reaction (*see* **Note 9**). High fidelity DNA polymerase or HotStarTaq DNA Polymerase should be used for the amplification of the target region. PCR was performed with the following conditions; 95 °C for 5 min, (94 °C for 30 s, 56 °C for 30s, and 72 °C for 30s) for at least 50 cycles, 72 °C for 7 min (*see* **Note 10**). You can also try SYBR Green 2× mixture if you have some difficulties to get a single band PCR amplicon.

3.9 Gel Electrophoresis

1. Run your PCR reaction on 2 % agarose gel prepared with 1× TAE buffer. Run 4 μl of the total PCR reaction (50 μl) on the gel. If you have a single band for your target region, you can use the rest of PCR reaction for the pyrosequencing reaction.
2. If you have some nonspecific bands in your gel results, you need to optimize your PCR conditions to get a single band. If you do not eliminate the unspecific bands, load whole PCR reaction into the gel and extract the desired band from the gel by using minElute Gel Extraction kit (Qiagen).

3.10 Preparation of Pyrosequencing Template

1. Bring all buffers and components at room temperature and incubate for 10 min.
2. In each well of 96-well PSQ plate, add 0.3 μM–0.4 μM sequencing primer in the presence of 40 μl of annealing buffer.
3. In 96-well PCR plate, add 36 μl of PCR reaction, 4 μl of Sepharose bead (undiluted), and 40 μl of 2×-binding buffers (*see* **Note 11**). Prepare two wells as a control (no DNA) for each internal sequencing primer.
4. Seal the plates and place it on microplate-shaker for 10 min at room temperature (1400 rpm or 250–300 × *g*).
5. Wash the vacuum tool with distilled water twice (fill the trough with water and place the vacuum tool inside, turn on the vacuum and wait for 30 s).

6. Remove PCR plate from shaker and immediately place filter tips of vacuum tool carefully into the PCR plate.
7. After capturing the DNA and beads on the filter tip of vacuum, you need to do several washes with the following solutions: 70 % EtOH, denaturation solution, and washing buffer.
8. Fill the trough with 70 % EtOH and place the vacuum tool into the trough. Turn on the vacuum and let it flush through the filters for 5 s.
9. Repeat **step 8** with denaturation buffer and washing buffer, 5 s each.
10. Hold the vacuum tool perpendicular and let it dry for 5 s, and return it to the horizontal position.
11. Close the vacuum pump (*see* **Note 12**) and place the filter tips which have only biotinylated DNA strand into the PSQ plate (prepared at **step 2**). Shake the vacuum gently few times and leave it for a few seconds inside the PSQ plate. This step releases the bound DNA strand from filter into the solution which has the sequencing primers and annealing buffer.
12. Annealing of primers to the DNA: incubate PSQ 96-well plate on 80 °C heat block for 2 min (not more than 2 min) and place it back on a cold surface.

3.11 Pyrosequencing

1. Put your PSQ plate in your instrument and insert the right cartridge gently.
2. Fill the cartridge by using the right concentration and volume of solution E (enzyme mixture), solution S (substrate mixture), and the four nucleotides dATP-a-S (A), dCTP (C), dGTP (G), and dTTP (T) according to the manufacturer's protocol (*see* **Note 13**) .

3.12 Run Sample and Data Analysis

1. For each pyrosequencing primer you are using for individual assay, you should enter the input sequence as a "sequence to analyze". Program automatically produces a dispensation order for your fragment.
2. For each sequencing primer, choice the well on the computer with the appropriate assay.
3. Click run button on the computer. Enzymes, substrate, and four nucleotides should be dispensed according to the predetermined order.
4. After the pyrosequence run, each well on the plate is shown with color with the following code:
   Blue wells = pass; Yellow or Orange wells = might be corrected with manual edit; Red wells = failed
5. Do not forget to check negative control wells in which you have only primer. You should not see any signal in those wells.

6. Pyro Q CpG Software will demonstrate two graphs at the end, one is for theoretical histogram and the second one is actual pyrogram showing the intensity of peaks. On both graphs, *x*-axes show the dispensation order of your target sequence. While *y*-axis shows number of nucleotides in the histogram, it shows the relative peak intensity in the pyrogram.
7. Since cytosine in the CG pair has two possible outcomes after bisulfite sequencing, it is represented as a gray bar with two nucleotides (T and C). "G" is not represented immediately after gray shaded "TC" because the dispensing order sometimes puts random nucleotides before "G".

   On top the gray boxes, calculated percentage of methylation is shown for each cytosine of CG pairs (Fig. 3).
8. You can trust the percentage of DNA methylation if the values are in the range of 5–95 % because of the 5 % deviation rate between the biological replicates.

4 Notes

1. There are three types of Hematoxylin Gill solutions: 1, 2, and 3. Their names are based on the concentration of the dye; Gill-1 has 2 g dye per 1 ml, Gill-2 has 4 g, and Gill-3 has 6 g per liter. Depending on the intensity of nuclear stain, you can choose one of these solutions. Protect these solutions from dye and store at room temperature. There is also Hematoxylin Mayer solution in which dye concentration is the half of the Gill-1 solution.
2. There is no true universal fixative, but 10 % neutral buffered formalin is the most commonly used fixative for the specimen preparation. Since it is carcinogenic and can cause eye, skin, and respiratory tract irritation, it should be prepared inside the hood.
3. Do not touch the sections and wax samples with your finger. Instead, use forceps or brush to pick up specimen from block face.
4. If you have difficulties to get one single smooth layer of section, cooling the surface of paraffin block on the ice might help. You can also try moisturizing the surface of the wax paraffin.
5. Pencil mark is permanent during the xylene and alcohol treatment. Numbering of each slide is important since we use first section as a reference slide for the next five or six sections. We cover the first section with glass coverslip in the presence of mounting media and use it for localization of the tumor cells during the laser capture microdissection. Do NOT cover the other slides.

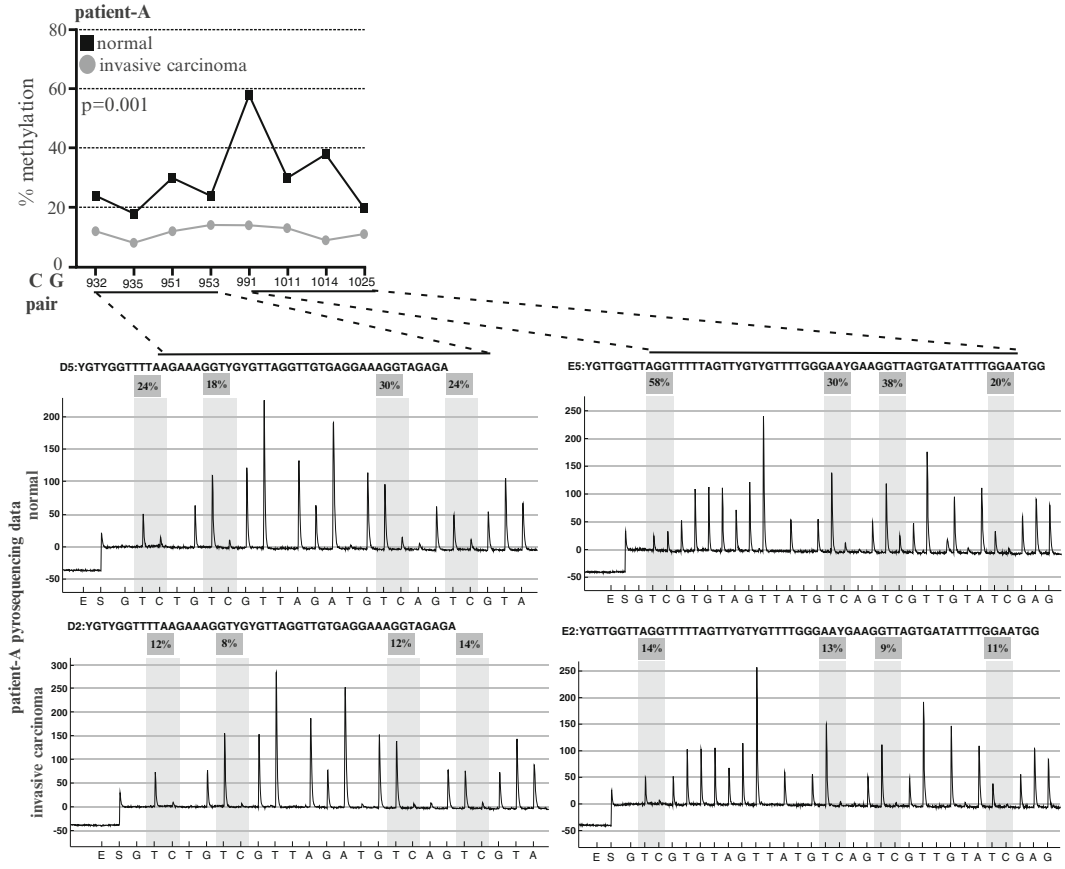

Fig. 3 Analysis of Pyrosequence Run. KIAA1199 (CEMIP) promoter region was amplified from bisulfite treated genomic DNA with two different primer pairs shown by two *solid lines* on the *y*-axis of the top graph. Upper pyrograms demonstrate the methylation profile of normal (benign) tissue; bottom pyrograms show the methylation profile of LCM dissected cancer cells. Whole methylation percentages are summarized on the top graph. Breast cancer patients have higher expression of KIAA1199 with respect to the normal (benign) tissue because of the hypomethylation on its promoter region

6. To collect the dissected cells from surface of the slide into the cap, the cap should be in close proximity to the surface. Be aware of the possible risk that the slide might get wet if the meniscus of the solution gets in touch with the surface of the slide.
7. One cell has approximately 57 μm^2 area on the surface of the slide. One single human cell with 2N karyotype has 6 pg DNA. Since the lower limit of Zymo research kit for bisulfite conversion is 500 pg, you need to isolate at least $500/6 = 84$ cells. Quality of DNA in FFPE tissue is very low, and therefore we multiply this amount with 10, so $84 \times 10 \times 57\ \mu m^2 = 48{,}000\ \mu m^2$ area should be selected as a minimum total "element" on one slide.

8. After the centrifugation, the solution might turn pinkish because of the hematoxylin and eosin staining of the cells. This color change could be a sign of presence of cell in the cap.
9. Treat 500 ng of unmethylated control DNA with EZ DNA methylation Gold kit. Use the MSP-control primer with bisulfite treated control DNA for the PCR reaction, and digest the PCR product with HpaII. If your bisulfate conversion works well, HpaII should not digest the PCR product.
10. Pyro-PCR should run at least 50 cycles to extinguish the extra nucleotides and primers in the PCR solution.
11. 20 μl PCR reaction from genomic DNA is usually enough for pyrosequencing reaction; however, you should increase the amount to 36–40 μl to increase the quality of signal because of the FFPE treated and laser captured DNA.
12. Closing the vacuum pump is very critical at this step; if you leave the pump on, you would suck up the primer solutions from the PSQ plate.
13. Since dATP can react with luciferin to produce photons, you should use modified dATP, called "dATPαS." It is not a substrate of luciferase and usage of the modified dATP reduces the noise ratio during the reaction.

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