In-situ sodium bisulfite modification of genomic DNA from microdissected melanoma paraffin-embedded archival tissues

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Summary

DNA methylation analysis of paraffin-embedded archival tumor tissues (PEAT) is important in clinical and translational research studies. Efficient identification and isolation of homogeneous cell population, optimal DNA extraction, and sodium bisulfite modification (SBM) are essential, particularly in small tumor lesions. Laser capture microdissection (LCM) coupled with an *in-situ* SBM improves the specificity, through histopathology accuracy, and the amount of genomic DNA modified for downstream methylation assays.

Keywords

melanoma; DNA methylation; laser capture microdissection (LCM); sodium bisulfite modification (SBM)

1. Introduction

Carcinogenesis and tumor progression process is triggered by a combination of genetic and epigenetic factors, which affect the expression of cancer-related genes (1,2). Epigenetic alterations - including non-coding RNA expression, histone modification, and DNA methylation - have been associated with melanoma development and progression (3,4). 5-methylation (5mC) and 5-hydroxymethylation (5hmC) of cytosine residues occur throughout the whole genome, and are often found in a sequence context of CpG dinucleotide (5,6). These epigenetic modifications have influence on gene expression in both normal and pathological status (7). DNA methylation can be assessed by several methodologies (8). However, DNA methylation patterns specific to tumor cells can be hidden by normal cells and tumor infiltrates such as leucocytes, endothelial cells, and fibroblasts. For example, melanoma is notoriously known for high level of tumor-infiltrating lymphocytes (TIL) which can significantly affect results of melanomas analyzed without previous microdissection of tumor cells. Therefore, it is essential to identify and analyze only specific and homogeneous cell types.

Laser capture microdissection (LCM) represents the most effective technology to isolate a morphologically homogeneous population of tumor cells, while maintaining genomic DNA integrity for downstream DNA methylation analysis (9). The combination of LCM and DNA methylation analysis is an essential requisite for melanoma PEAT analysis, which attempt to identify melanoma-related genomic and epigenomic (DNA methylation) aberrations.

Sodium bisulfite modification (SBM) of genomic DNA is the most commonly used method for discrimination of methylated and unmethylated alleles (10). This is also a start-point conversion for several methodologies, including PCR amplification (MSP) and BeadChip array hybridization (8). One problem of SBM is the high loss of the initial genomic DNA amount (11). This inconvenience, along with the loss of genomic material during extraction, collectively requires high volumes of starting paraffin-embedded archival tumor tissues (PEAT). In the assessment of primary tumors and micrometastasis to lymph nodes the availability of melanoma PEAT is often limited, which poses a major problem for downstream analysis. To reduce the loss of sample DNA our laboratory, originally designed a SBM of tissue slides without the requirement of DNA isolation (12). This protocol has been modified to cells collected by LCM, where SBM is performed while the captured cells are still attached to the LCM-cap. This coupled protocol has proven to decrease genomic DNA loss in colorectal and breast carcinoma studies (4, 13). In this chapter, we describe the workflow to couple LCM and in-situ SBM of melanoma PEAT specimens.

2. Materials

Prepare and store all reagents at room temperature (unless indicated) using ultrapure molecular grade water and reagents. Follow all waste disposal regulations when disposing biological waste materials.

2.1. PEAT processing and LCM

- a. Superfrost non-coated slide (VWR # 48311-600)
- b. Xylene (Fisher # HC-700-1Gal)
- c. Ethanol (Fisher # HC-800-1Gal)
- d. Gill's hematoxylin (Harleco # 65065)
- e. Eosin Y (Harleco # 592-75)
- f. CapSure® Macro LCM Caps (LCM0211, Applied Biosystems, Life Technologies)
- g. Veritas™ or other Arcturus microdissection instruments (Applied Biosystems, Life Technologies)

2.2. Sodium bisulfite modification (SBM)

- a. Sodium bisulfite (SB) solution: mix 1.5 mL of 3M NaOH (Fisher # S318-500), 165 mg of hydroquinone (Sigma # H9003), 5.7 g of sodium metabisulfite (Sigma # S9000) and H₂O to 12 mL (see Note 1). Check that the pH is 5.0.

- b. Proteinase K (PK) solution: mix 2 μ L of 20 mg/mL proteinase K (Qiagen # 19133) and 50 μ L of TE buffer pH 8.0 (Sigma # 93283).

2.3. Methylation analysis

- a. PerfeCTa® SYBR Green FastMix for iQ (Quanta BioScience # 95071-250)
- b. CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories #185-5196)
- c. Bio-Rad CFX Manager 2.0 (Bio-Rad Laboratories #184-5000)

3. Methods

3.1. PEAT processing and LCM

1. Slide preparation
 - a. Cut with microtome tissue sections of 5–8 μ m of thickness (see Note 2).
 - b. Mount the sections onto Superfrost non-coated slides.
 - c. Perform deparaffinization, rehydration, hematoxylin staining and then dehydration of the sections.
 - d. Hematoxylin and eosin staining for the reference slide (see Note 3).
 - e. Have surgical oncology pathologist mark tissue area of interest in a reference slide.
2. Laser capture microdissection
 - a. Place the sample and the reference slides with tissue section into the LCM instrument.
 - b. Place CapSure macro caps on tissue section (Fig. 1a).
 - c. Based on the reference slide, select the cells of interest in the sample slide and pulse the infrared laser for targeted cells capture (Fig. 1b).
 - d. Remove cap with adhered target cells (Fig. 1c).

3.2. Sodium bisulfite modification (SBM)

1. DNA denaturation
 - a. Add 200 μ L of 0.2 M NaOH into 0.6 mL microcentrifuge tubes (one tube per LCM-cap; Fig. 1d).
 - b. Place the LCM-caps on the tubes and invert the tubes.

¹SB solution needs to be freshly prepared. First add 6 mL of H₂O into a 15 mL tube, then add NaOH and finally hydroquinone and sodium metabisulfite. Cap the tube loosely and heat the solution in a 60°C water bath until the solutes are dissolved. Finally, add H₂O to 12 mL.

²The recommended tissue thickness is 8 μ m; however, it can be decreased to 4 μ m to avoid contamination of normal tissue layers under tumor tissue.

³In order to select the desired population of cells, tissue slides can be stained by hematoxylin and eosin or special staining and immunohistochemistry techniques.

- c. Centrifuge the inverted tubes for 1 min $\times$ 1000 rpm (see Note 4).
 - d. Incubate for 15 min at 37°C.
 2. DNA sulphonation and hydrolytic deamination
 - a. After incubation, turn tubes back to upright position.
 - b. Add 400 μ L of SB solution into new 0.6 mL microcentrifuge tubes.
 - c. Transfer the LCM-caps from the DNA denaturation 0.6 mL tubes to the new 0.6 mL tubes (see Note 5).
 - d. Invert the new tubes and centrifuge for 1 min $\times$ 1000 rpm (see Note 4).
 - e. Incubate for 8 h at 60°C.
 3. DNA alkali-desulphonation
 - a. After incubation, turn tubes back to upright position.
 - b. Add 200 μ L of 0.3 M NaOH into new 0.6 mL microcentrifuge tubes.
 - c. Uncap the tubes and rinse each LCM-cap twice with 1 mL preheated 50°C H₂O. Transfer the LCM-caps to the new 0.6 mL tubes.
 - d. Invert the new tubes and centrifuge for 1 min $\times$ 1000 rpm (see Note 4).
 - e. Incubate for 15 min at 37°C.
 4. Deproteinization
 - a. After incubation, turn tubes back to upright position.
 - b. Add 52 μ L of PK solution into new 0.6 mL microcentrifuge tubes.
 - c. Uncap the tubes and rinse each LCM-cap once with 1 mL preheated 50°C H₂O. Transfer the LCM-caps to the new 0.6 mL tubes.
 - d. Invert the new tubes and centrifuge for 1 min $\times$ 1000 rpm (see Note 4).
 - e. Incubate for 24 h at 50°C.
 5. Proteinase K inactivation
 - a. After incubation, turn tubes back to upright position.
 - b. Centrifuge 1 min at 1000 rpm the tubes with LCM-caps still in place (see Note 6).
 - c. Incubate 15 min at 95°C.
 - d. Store vials with lysate at -20°C until use (see Note 7).

⁴Centrifugation should remove all air bubbles from the surface of the LCM-cap.

⁵During this step, keep each tube lightly squeezed when putting the cap on. This will create slight under pressure, allowing for remaining air to expand during incubation.

⁶All tissue and fluid should be released from the LCM-cap and reach the bottom of the tube.

⁷Modified DNA can be stored at 4°C for use within 24 h, or at -20°C for storage up to 6 months.

3.3. Methylation analysis

The methylation status of the processed DNA can be analyzed by different methodologies. Here we indicate how to perform quantitative methylation-specific PCR amplification.

- a. SBM-DNA is subjected to PCR amplification in two reactions using two sets of primers. One specific for methylated (M) alleles and the other specific for unmethylated (U) alleles.
- b. The quantitative amplification of M and U alleles is performed in a final reaction volume of 20 μ L containing 10 μ L of PerfeCTa® SYBR Green FastMix for iQ (see Note 8), 10 μ g of BSA, 0.2 μ M primers (M or U), DNA template (lysate) and H₂O.
- c. PCR amplification and detection are carried out in a CFX96 Touch™ Real-Time PCR Detection System and the Ct values are extracted using the CFX manager 2.0 (see Note 9).
- d. The relative methylation level of each target is calculated using the 2^{-Ct} method. Difference between Ct value for M and U allele is established ($Ct = Ct_M - Ct_U$) and is used as exponent (n) in the equation 2^{-n} . The result represents the relative number of methylated alleles versus unmethylated alleles (Fig. 1d).

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⁸Perfecta IQ contents: 2X reaction buffer containing optimized concentrations of MgCl₂, dNTPs (dATP, dCTP, dGTP, dTTP), AccuFast Taq DNA Polymerase, SYBR Green I dye, 20 nM fluorescein, and stabilizers.

⁹Optimization of PCR conditions is important to obtain accurate results. For optimization of each set of primers methylated and unmethylated controls are needed (13,14).

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Figure 1.

In situ-SBM algorithm. (a) Positioning of LCM-cap on tissue section. (b) Selection of cells of interest and pulse of infrared laser. (c) Separation of cells bounded to the LCM-cap polymer. Light micrographs show melanoma nested in heterogeneous tissue prior LCM (d), after LCM (e) and the LCM-cap containing the adhered targeted melanoma tissue sections (f). Black arrowheads indicate the targeted melanoma tissue. (g) Diagram of a microcentrifuge tube containing the LCM-cap with the isolated population of cells. (h) CFX manager 2.0 output view of the quantitative PCR amplification for methylated (M) and unmethylated (U) alleles.