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Whole-Genome Allelotyping Using Laser Microdissected Tissue

Colleen M. Feltmate and Samuel C. Mok

Summary

Laser-based microdissection technologies have been recently developed and applied to procure homogenous populations of tumor cells from paraffin-embedded and frozen tissue sections. When combined with whole-genomic amplification techniques, sufficient amounts of DNA can be generated from a small number of tumor cells procured by laser-based microdissection. Amplified DNA can then be used to perform high-throughput genome-wide allelotyping using fluorescent-labeled microsatellite markers spanning the whole genome. Loss of heterozygosity can be assessed by Genescan and Genotyper software (ABI Prism).

Key Words: Allelotyping; loss of heterozygosity; ovarian cancer.

1. Introduction

Loss of heterozygosity (LOH) has been widely used in an effort to identify tumor-suppressor genes in various tissue types ***(1–4)***. This technique utilizes markers specific for a particular chromosomal locus, which can differentiate both paternal and maternal alleles in normal tissue. The heterozygous pattern identified in normal non-neoplastic tissue is then compared with that of neoplastic or tumor DNA from the same patient. LOH is identified if one of the heterozygous alleles is missing.

Classical LOH studies used DNA extracted from whole-tissue samples ***(3,5,6)***. Several studies have also used gross microdissection techniques in an effort to enrich specific cell populations of interest ***(7,8)***. Contamination by stromal tissue, however, may still skew LOH patterns, decreasing sensitivity in the detection of loss of heterozygosity. To circumvent this problem, microdissection techniques, which can procure precisely defined homogenous tumor

From: *Methods in Molecular Biology, vol. 293: Laser Capture Microdissection: Methods and Protocols*
Edited by: G. I. Murray and S. Curran © Humana Press Inc., Totowa, NJ

cell population with low or even no contamination by nontumor cells, are required. Two laser-based microdissection systems have recently been developed. The PixCell II Laser Capture Microdissection (LCM) system (Arcturus Engineering, Mountain View, CA) uses direct contact with a transparent ethylene vinyl acetate (EVA) thermoplastic film to procure specific cells of interest ***(9,10)***. The other (Leica, Germany) uses a frame foil slide and laser cutting technique. These laser-based microdissection techniques can be applied to both paraffin-embedded and frozen tissue sections. However, when only a small number of cells are available, the amount of DNA isolated may not be sufficient for large-scale allelotyping studies. Through whole-genome amplification methods ***(11,12)*** the entire genome can be amplified. High-throughput allelotyping can then be performed using fluorescent-labeled microsatellite markers spanning the whole genome ***(1,2)***.

2. Materials

2.1. Tissue Processing

1. OCT compound.
2. Formalin.
3. Ethanol.
4. Xylene.
5. Paraffin wax.
6. Cryomold.
7. Liquid nitrogen.
8. Cryostat and microtome.
9. Uncoated glass slides.
10. Polyethylenenaphthalate (PEN) framed foil slides (Leica, Germany).
11. 1% Methyl green.

2.2. Microdissection and DNA Extraction

1. PixCell II LCM System (Arcturus Engineering).
2. LMD-Laser Microdissecting System (Leica, Germany).
3. 0.5-mL Eppendorf tube.
4. Digestion buffer: 1X Expand High Fidelity Buffer (Roche), 4 mg/mL proteinase K, and 1% Tween-20 at 55°C.

2.3. Whole-Genome Amplification

1. Reaction mixture: 0.05 mg/mL gelatin, 40 m*M* 15-mer random primers (Operon Technologies, Alameda, CA), 0.2 m*M* dNTP, 2.5 m*M* $MgCl_2$, 1X Expand High Fidelity Buffer, and 3.5 U of Expand High Fidelity Polymerase (Roche).
2. Thermocycler.

2.4. Allelotyping (Applied Biosystems, Foster City, CA)

1. ABI Prism Linkage Mapping Set.
2. True Allele Buffer (10X GeneAmp PCR buffer II, GeneAmp dNTP mix [2.5 m*M*], AmpliTaq Gold DNA Polymerase [5 U/mL], 25 m*M* $MgCl_2$).
3. ABI310-Genetic Analyzer (Applied Biosystems).
4. 1X Buffer.
5. POP-4 Polymer.
6. Capillary (47 cm × 50 μm).
7. Rox standard.
8. Deionized formamide.
9. Genescan and Genotyper software (Applied Biosystems).

3. Methods

The methods described below outline (1) tissue preparation, (2) microdissecting procedures, (3) extraction of DNA, (4) whole-genome amplification procedures, and (5) the allelotyping process.

3.1. Tissue Preparation

Tissue processing is initiated within 1 h of surgical removal from patients. Samples can be snap-frozen in liquid nitrogen or fixed in formalin. The two types of processing technologies are described below.

3.1.1. OCT Preparation

Tissue is cut into 0.5-cm^3 sections and placed centrally in a plastic cryomold. OCT compound is then used to fill the mold. The entire cryomold is placed on the surface of liquid nitrogen until OCT is solidified. Tissue blocks can then be stored in a –80°C freezer.

3.1.2. Formalin Fixation

Tissue is placed in formalin overnight on a rocking platform. Tissue is dehydrated through an ascending series of ethanol, cleared in xylene, and embedded in paraffin.

3.1.3. Tissue Sectioning and Staining

1. Frozen sections (7 μm) are cut using a cryostat and mounted on either a plain slide or a PENfoil slide.
2. They are immediately fixed in 70% ethanol for 30 s, stained with 1% methyl green (*see* **Note 1**) for 5 s, and washed in distilled water.
3. Sections on plain slides are dehydrated through an ascending series of ethanol: 70%, 30 s; 95% × 2, 30 s; 100% × 2, 5 min.

4. Sections are then cleared in xylene for 5 min and allowed to air-dry.
5. Sections on PENfoil slides are fixed in a similar manner. The dehydration process utilizes 70% and 95% ethanol for 30 s each.
6. Paraffin sections (7 μm) are cut using a microtome.
7. Sections on plain slides are dewaxed by routine methods in xylene and ethanol.
8. Staining and dehydration are performed similarly as above.
9. Sections on PENfoil slides are dewaxed in xylol for 2 min.
10. A descending series of ethanol is used: 100% × 2, 30 s; 95% ×2, 30 s; 70%, 10 s; dH_2O, 10 s. Stain as above, wash in dH_2O, and air-dry sections.

3.2. Microdissection and DNA Extraction

3.2.1. PixCell II LCM System

1. Sections utilizing plain glass slides are required for the PixCell II LCM system.
2. This microscope focuses the laser beam to discrete spot sizes (7, 15, or 30 μm), delivering precise pulsed doses that are controlled by the operator, to the targeted film. Targeted cells are transferred to the cap surface (**Fig. 1**).
3. The operator must control both the movement of the stage and the laser firing.
4. A special film adhesive is applied to the slide prior to microdissection to remove poorly bound tissue fragments.
5. The cap should be reviewed periodically during dissection, using a blank slide, to ensure there is no contamination by unprocured tissue fragments.
6. Approximately 5000 cells are procured for the digestion process (*see* **Note 2**).
7. After completing the dissection, the cap can be placed directly into an Eppendorf tube for DNA extraction.

3.2.2. Leica LMD System

1. This laser microdissecting system requires the use of special PENfoil slides. Once the specific magnification is determined, the cells of interest are circumscribed on the screen image and then cut automatically.
2. The laser beam moves over the specimen to make the cut.
3. The specimen remains stationary so that it can be clearly observed during the cutting process.
4. The material cut out by the laser falls into the cap of an Eppendorf tube below (**Fig. 2**).
5. There is an inspection mode to visually confirm that the dissected cells have been deposited in the tube.

3.2.3. DNA Extraction

1. Fifty μL of digestion buffer are added either directly to the Eppendorf tube and inverted for the PixCell cap dissection or directly to the cap containing the dissected material from the Leica dissection process.
2. The digestion takes place at 55°C over 72 h in a humidified incubator.
3. After 24–36 h, an additional microliter of 20 mg/mL proteinase K solution is added to ensure complete digestion.

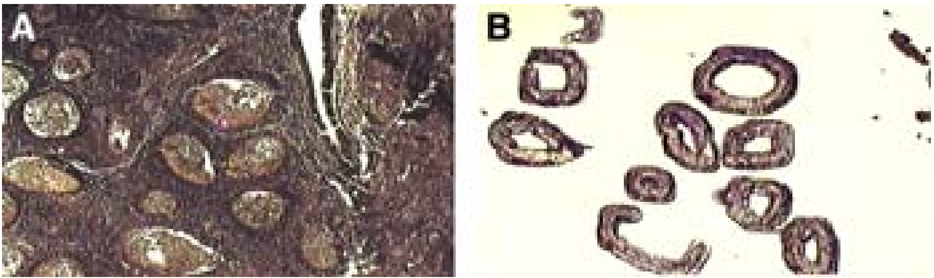

Fig. 1. Laser capture microdissection of mucinous epithelial ovarian tumor **(A)**. Tumor cells procured on cap are shown in **(B)**.

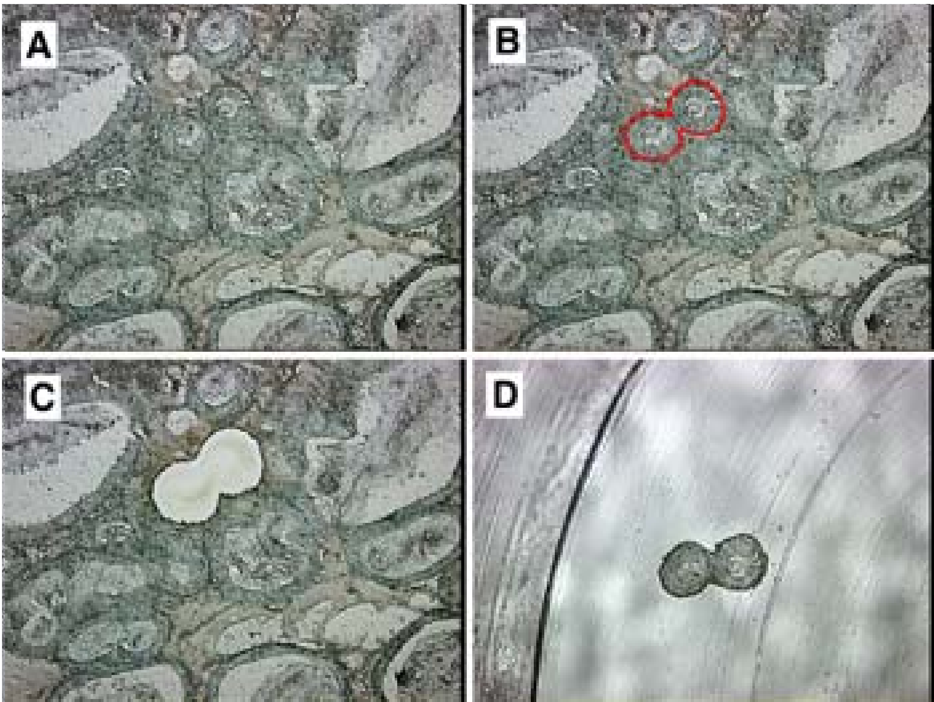

Fig. 2. Photomicrograph of mucinous epithelial ovarian tumor **(A)**. Tumor cells selected are outlined by red line **(B)**. After laser cut **(C)**, cut section in Eppendorf cap **(D)**.

4. The digest is then spun briefly, heated to 95°C for 10 min to inactivate proteinase K, spun briefly again before transferring to a new tube, and stored at 4°C (*see* **Notes 3** and **4**).

3.3. Whole-Genome Amplification

1. Whole-genome amplification is performed by using a modified primer-extension preamplification (I-PEP) process ***(12)***.
2. Ten μL of digested microdissected DNA is added to 50 μL of the I-PEP reaction mixture.
3. Fifty primer extension cycles are carried out in a thermocycler after an initial 94°C, 3 min denaturation step.
4. Each cycle consists of a 1-min at 94°C, 2 min at 37°C, a ramping step of 0.1°C/s up to 55°C, a 4-min primer extension step at 55°C, and 30 s at 68°C.
5. Following amplification the I-PEP DNA can be quantified using a fluorometer. I-PEP product can be diluted to 50 ng/μL and stored at –20°C.

3.4. Allelotyping

1. Microsatellite markers are obtained from ABI Prism Linkage Mapping Sets. These sets may vary in average interval between loci, e.g., 5 or 10 cM, and are optimized for high-throughput allelotyping.
2. Microsatellite markers consist of fluorescent primer pairs end-labeled with fluorochromes FAM, VIC, or NED that amplify dinucleotide repeat fragments.
3. Cytogenetic location of the markers can be determined by using the following websites: UCSC's Genome Browser (http://genome.ucsc.edu), NCBI's Map Viewer (http://www.ncbi.nlm.nic.gov/genome/guide) or Ensemble (http://www.ensembl.org) (*see* **Notes 5–7**).

3.4.1. PCR Reactions

1. PCR reactions are carried out in a 10-μL volume utilizing 1 μL of I-PEP DNA, 6.6 μL of True Allele Premix, 2.54 μL dH_2O, and 0.66 μL of primer pair.
2. Thermocycler settings for amplification are as follows: 95°C for 12 min; 10 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 30 s; 30 cycles of 89°C for 15 s, 55°C for 15 s, 72°C for 30 s.
3. PCR product is then stored in a –20°C freezer until ready for the pooling process.

3.4.2. Pooling PCR Products

1. PCR products for each sample can then be pooled by panel such that the fluorescent primer products are pooled together by volume in a ratio of 1:1:2 (HEX:VIC:NED).
2. A stock solution of formamide and Rox standard is then made using 12 μL of formamide and 0.55 μL Rox for each sample (this solution can be stored for 2 wk in a –20°C freezer).
3. Two μL of pooled fluorescently labeled PCR product is then added to 12.5 μL of the formamide-Rox stock solution.
4. The mixture is then heated to 95°C for 5 min, cooled on ice, and placed in the ABI310-Genetic Analyzer for data acquisition.

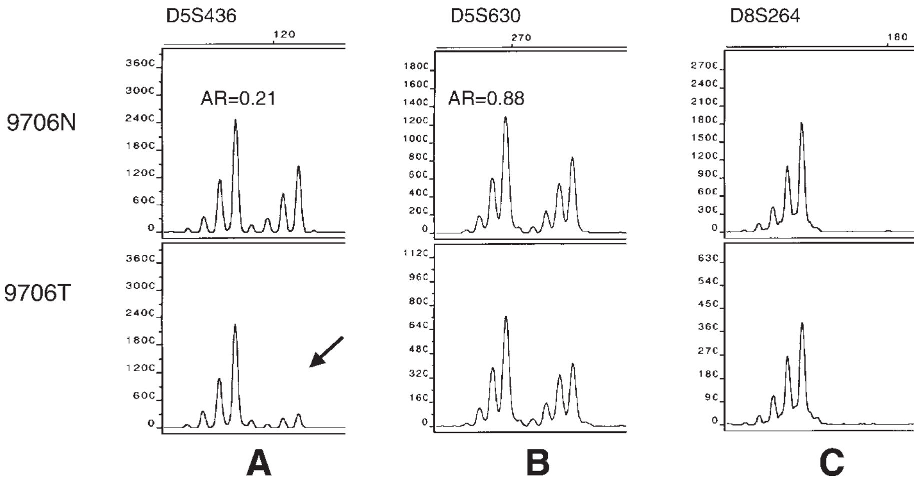

Fig. 3. Representative example electropherogram traces for normal (9706N) and tumour (9706T) genotypes. Allelic ratios (AR) were calculated as described and are shown at the top. **(A)** Arrow, LOH for marker D5S436. **(B)** marker D5S630 shows heterozygous without loss and **(C)** marker D6S264 noninformative NI or homozygous.

3.4.3. Data Acquisition and Analysis

1. Data are initially processed using Genescan 2.1 software.
2. Result files can then be imported into Genotyper (version 2.5) and the data tabulated according to allele size, allele peak height, and allele area.
3. An allelic ratio is calculated using allelic peak volume in a formula: (*N1/N2*)/(*T1/T2*), where *N* is normal allele and *T* is tumor allele.
4. For each particular marker locus, LOH is assessed in the corresponding tumor sample if it is informative (heterozygous) in the normal DNA sample. LOH is present when the decrease in one allele is greater than 50% (normal/tumor allelic ratios <0.5 or >2.0).
5. Individual results are classified into homozygous, heterozygous with no loss, and heterozygous with loss. **Figure 3** demonstrates the electropherogram traces for various allelic patterns.
6. Software package developed by the University of Texas Southwestern Medical Center supports a clustering algorithm as well as basic calculations for percent LOH and fractional allelic loss ***(1,13)***.

4. Notes

1. The choice of staining depends on the tissue type under study. It is important to note that hematoxylin may affect the DNA template such that subsequent PCR yield may be compromised ***(14)***.

2. In order to eliminate loosely bound contaminate tissue from the slide, a Prep Strip (Arcturus) can be first applied to the slide. If contamination is noted on the cap itself, a CapSure Pad (Arcturus) can be used to remove the unprocured tissue fragment(s).
3. DNA quality may vary in archival formalin fixed tissues. Both quality and quantity of DNA may require increased amount of dissected tissue prior to DNA digestion in order to provide adequate DNA template for subsequent reactions.
4. Digestion of microdissected tissues can be accomplished over different time courses. We prefer a 72-h protocol with the addition of 1 mL of proteinase K (20 mg/mL) after 24 h.
5. Both PCR reaction and pooling for allelotyping are labor-intensive. Robotics can increase both accuracy and speed in these processes.
6. ABI provides pooling panels for their fluorescent markers. The majority of times these can be followed, however, we do not recommend pooling more than eight markers especially when DNA isolated from formalin-fixed tissue is utilized. When making decisions about pooling, consideration should be given to the dye color, fragment size, and specific loci of interest.
7. Although ABI recommends that each capillary can be used for 100 reactions, we are able to obtain reliable electropherograms through 300 reactions.

Acknowledgments

This study was supported by Ovarian Cancer Research Program grant no. DAMD17-99-1-9563 from the Department of Defense, Early Detection Research Network Grant CA86381 from the National Institute of Health, Department of Health and Human Services, Gillette Center for Women's Cancer, Adler Foundation, Inc., the Ovarian Cancer Research Fund, Inc., the Morse Family Fund, and the Natalie Pihl Fund, and DF/HCC Ovarian Cancer SPORE program.

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