Tissue preparation for laser capture microdissection and RNA extraction from fresh frozen breast tissue

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For decades, tissue heterogeneity represented a challenge for scientists wishing to study isolated cells or cell populations. Traditional methods of selective purification (e.g., MagAB) are compromised by tissue artifacts and contamination and limited by a minimum tissue-requirement volume. The application of laser capture microdissection (LCM) technology to facilitate selective sampling of individual cells or groups of cells from histological specimens is gaining popularity and is now an established method of procuring cells for many downstream RNA, DNA, and protein experiments (1–7). Although groups have reported successful RNA extraction from formalin-fixed, paraffin-embedded tissue (8), fresh frozen tissues (FFT) are recommended for optimal RNA recovery. However, manipulation of FFT can be extremely challenging, and RNA purity and yield are dependent on optimal tissue preparation.

Despite the increasing acceptance and application of LCM, there seems to be no consensus regarding tissue preparation prior to LCM. Literature review has indicated that variations in tissue preparation methods can compromise the quality of RNA by up to 75%. The objective of this study was to determine the effects of tissue manipulation on the quantity and quality of RNA by comparing available protocols and to define a tissue preparation process that facilitates optimal LCM without affecting RNA quality.

With the approval of our institutional review board, a mastectomy tissue specimen was obtained immediately after surgery, cut into blocks, washed briefly with ice-cold phosphate-buffered saline (PBS), pH 7.2, followed by ice-cold isotonic (0.25 mol/L) sucrose, embedded in optimum cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and stored at -80°C. To determine the specimen's baseline RNA quality, a core biopsy was taken from one FFT block and RNA was extracted (Stratagene Absolutely RNA® Microprep kit). Four different hematoxylin and eosin (H&E)-based tissue preparation protocols that reported successful extraction of quality RNA from FFT were identified in the literature. These four protocols (protocols 2, 3, 5, and 6) were compared with an H&E-based protocol developed in our laboratory—protocol no. 4—which was optimized for minimal preparation time with adequate preservation of histomorphology and RNA integrity. In addition, in order to determine any potential RNA compromise from H&E staining, we included a methyl green (MG)-based protocol (protocol no. 1) (Table 1). Eight 8-µm serial sections were cut (-25°C) from the same tissue block for each protocol and placed onto polyethylene naphthalate membrane slides.

Immediately after sectioning, slides were stained with fresh solution and microdissected. The PALM Laser MicroBeam system (P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany) was used for this study. This system employs a high-energy laser beam to microdissect along a precise, predefined line and to catapult area-of-interest samples from the slide into a collection cap containing 6 µm solution [20 µL 0.5 M EDTA pH 8.0, 2000 µL 1 M Tris, pH 8.0, 50 µL Igepal® Ca 630 (Sigma-Aldrich, St. Louis, MO, USA), and 97 3 mL diethylpyrocarbonate (DEPC)-treated double-distilled water]. A total area of 250,000 µm^2 was microdissected from each slide, yielding approximately 2,000,000 µm^2/protocol. Each 2,000,000-µm^2 area was thought to represent approximately 35,000 cells (one cell is approximately 57 µm^2 in area) and 0.35 µg total RNA (one cell is approximately 0.01 ng RNA). The cap was immediately placed on a microcentrifuge tube containing 12.5 µL lysis buffer (one cap per slide), which was inverted, vortex mixed, and stored upside down at -80°C. Using the Stratagene microprep kit, pure

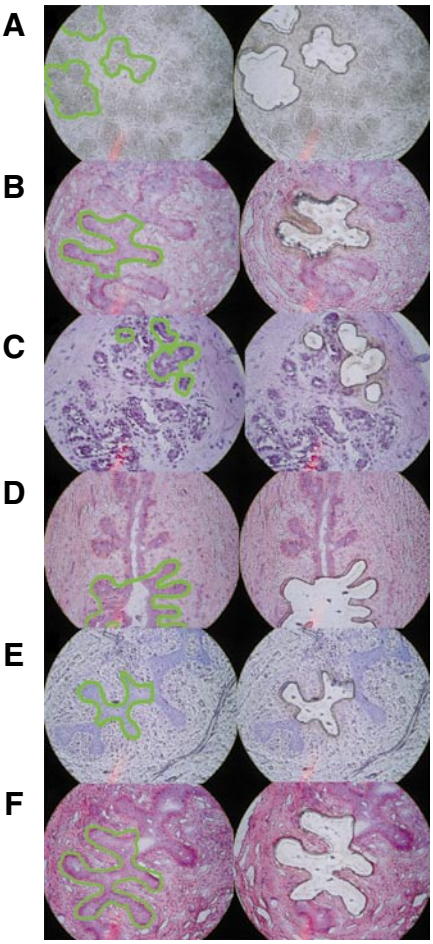

Figure 1. Breast tissue sections from different preparation protocols used for laser capture microdissection (LCM). Representative photomicrographs (40× magnification) from tissue preparation (A) protocol 1 and (B–F) protocols 2–6 [1, methyl green; 2–6, hematoxylin and eosin (H&E)] demonstrating a region of normal breast epithelium demarcated for laser microcapture dissection on the left, and after dissection/collection of cells on the right.

Table 1. Staining Protocols

Protocol No.	1	2[a]	3[b]	4	5[c]	6[d]
Stain Slides	Together	Individually	Individually	Individually	Individually	Individually
SlideTemp	RT	RT	Cooled	Thawed	Thawed	RT
Reagent Temp	RT	RT	Cooled	On Ice	RT	RT
Thaw	5 min	–	20 s	60 s	30 s	–
70% Alcohol	30 s	30 s	3 min	30 s	–	Dip
75% Alcohol	–	–	–	–	30 s	–
Double-Distilled Water	3 × 10 s	10 s	Dip	Dip	30 s	Dip
MethylG	10 s	–	–	–	–	–
Mayers	–	10 s	3 min	10 s	–	1 min
Special Stain	–	–	–	–	100 µL, 20 s	–
Double-Distilled Water	–	10 s	3 min	10 s	30 s	Dip
Ammonia Water	–	15 s	–	10 s	–	60 s
100% Alcohol	–	–	–	–	–	–
95% Alcohol	–	–	–	–	–	Dip 70
70% Alcohol	–	15 s	–	10 s	–	Dip 95
Eosin Y	–	15 s	30 s	10 s	–	1 min
70% Alcohol	30 s	–	Dip	–	–	–
75% Alcohol	–	–	–	–	30 s	–
95% Alcohol	30 s	15 s	Dip	10 s	30 s	Dip
95% Alcohol	30 s	15 s	–	20 s	–	Dip
100% Alcohol	30 s	15 s	Dip	30 s	30 s	Dip
100% Alcohol	30 s	30 s	–	60 s	–	Dip
Xylene	3 × 30 s	5 min	–	45 s	5 min	1 min
Air	10 min	Not Specified.	10 min	5 min	–	3 min
Total Preparation Time	20 min	8 min[e]	~20 min	10 min	9 min	~9 min
Slide Storage	-80°C	-80°C	-80°C	-80°C	-80°C	-80°C
Preservation of Histomorphology[f]	–	+	+	+++	+	++
Efficiency of Microdissection[g]	+	++	+	+++	++	+

RT, room temperature; Mayer's, Mayer's hematoxylin.
[a]Data from The University of Alabama at Birmingham laser microdissection facility, lcm.path.uab.edu/protocols.htm, accessed May 2006.
[b]Data from P.A.L.M. Microlaser technologies, www.palm-microlaser.com, accessed May 2006.
[c]Data from Arcturus; www.arcturus.com/research_portal/products/histo_main.htm, accessed May 2006.
[d]Data from National Cancer Institute molecular profiling initiative; cgapmf.nih.gov/Protocols/Slides/SlideProtocols/ImmunoLCM.html, accessed May 2006.
[e]This time does not include the air-dry time.
[f]Assessed based on the ability to distinguish between various cell types, such as epithelial cells, myoepithelial cells, stromal cells, endothelial cells, and lymphocytes after staining, with particular attention being placed on the ability to distinguish between the epithelial and myoepithelial layers of the breast tissue.
[g]Assessed in terms of the ability of the laser to (*i*) precisely dissect around the cells of interest without compromising the integrity of the cells, and (*ii*) transfer the cells of interest into the capture device.

RNA from each sample was eluted and captured in a microcentrifuge tube (final volume of 30 µL). Additional DNase treatment was also performed.

The quality and quantity of isolated total RNA were assessed using the RNA 6000 Pico LabChip kit and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Five hundred fifty microliters RNA 6000 Pico gel matrix were placed on a spin filter, centrifuged at maximum speed for 10 s, and divided in 65-µL aliquots. To each aliquot, 1 µL RNA 6000 Pico dye was added, and the mixture was vortex mixed and centrifuged at maximum speed for 10 min. Using the priming solution, each aliquot was filled with gel-dye mix, conditioning solution, marker, 1 µL RNA 6000 ladder, and RNA samples, and was then vortex mixed for 1 min before being run on the Bioanalyzer. Both the 28s/18s rRNA subunit ratio and the RNA integrity number (RIN) were used to evaluate RNA integrity.

Comparing MG to H&E, H&E allowed for faster and more exact identification of cells of interest (Figure 1, A–F). Methodological differences between the various H&E protocols resulted in great variation in preservation of histomorphology (i.e., the

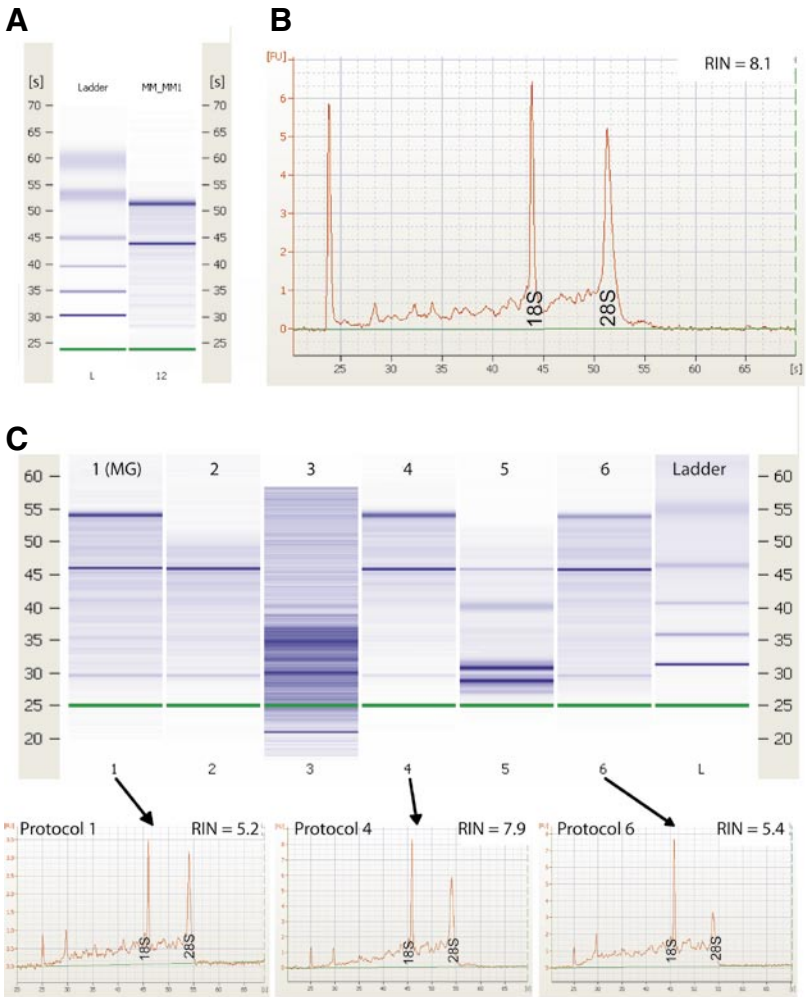

Figure 2. Baseline RNA quality control of samples obtained from different tissue preparation protocols using laser capture microdissection (LCM). A core biopsy sample was taken from the fresh frozen tissue block, and RNA was isolated. Using the RNA 6000 Pico LabChip kit and the 2100 Bioanalyzer, RNA quality was (A) assessed by gel and (B) plotted. RNA quality by different tissue preparation methods is shown in gel (C) and plot (below, at arrows) format. Each gel lane shows the RNA extracted from cells of microdissected tissue selections prepared using staining protocols 1–6. The corresponding RNA integrity numbers for the samples with the highest quality RNA are illustrated (rows 1, 4, and 6). S, sample; L, ladder; FU, frequency units; 18S, 18S band; 28S, 28S band; MG, methyl green; RIN, RNA integrity number.

ability to distinguish between various cell types, such as epithelial cells, myoepithelial cells, stromal cells, endothelial cells, and lymphocytes). The protocol developed in our laboratory (protocol 4) allowed for optimal identification of these cellular components, particularly the ability to distinguish between the epithelial and myoepithelial layers of the breast tissue. The efficiency of microdissection was assessed in terms of the ability of the laser to (*i*) precisely dissect around the cells of interest without compromising the integrity of the cells, and (*ii*) transfer the cells of interest into the capture device. Variations in staining protocols (i.e., different concentrations of ethanol, durations at each incubation, and varied exposure time to xylene and air) affected the efficiency of microdissection primarily due to variable water content of the slide after staining. When assessed for efficiency, protocol 4 allowed for optimal microdissection of the histological specimens.

Variations in tissue preparation also affected both quality and quantity of RNA yield. The expected yield for each protocol was 0.35 µg RNA. The observed total RNA yield ranged from 28.3% to 97.1% of expected (0.10–0.34 µg) (Table 2). The baseline RNA quality (i.e., prestaining) was 8.1. The RNA quality poststaining ranged from 2.3 to 7.9 with only three samples yielding RNA of adequate quality that could be used for downstream applications. The RNA isolated from the cells prepared using protocol 4 was of the highest quality and showed minimal degradation when compared with

Table 2. RNA Yield of LCM Samples Obtained from Different Tissue Preparation Protocols

Sample ID	ng/µL	A260	A280	A260/A280	A260/A230	Volume (µL)	Total RNA (µg)
Core[a]	49.71	1.263	2.03	2.03	1.99	20	1.0
1	3.38	0.084	0.05	1.71	0.14	30	0.10
2	5.57	0.139	0.093	1.5	0.16	30	0.17
3	5.14	0.129	0.051	2.54	0.21	30	0.15
4	11.37	0.284	0.156	1.83	0.27	30	0.34
5	6.13	0.153	0.078	1.97	0.26	30	0.18
6	7.53	0.188	0.091	2.07	0.45	30	0.23

An estimated 0.35 µg total RNA was isolated from each protocol. RNA isolated from the cells prepared using protocol 4 were of highest yield and showed minimal degradation when compared with expected yield (total RNA = 0.34 vs. 0.35 µg). LCM, laser capture microdissection.
[a]Denotes the tissue sample prior to slide preparation.

baseline RNA quality. (RNA integrity number, 7.9 vs. 8.1; 98% recovery). Overall, the protocol developed in our laboratory (protocol 4) was optimal.

Sophisticated high-throughput RNA-based experiments, such as gene-expression microarrays, have significantly enhanced our knowledge of cancer biology, and we now appreciate that carcinogenesis is a complex, multi-step process with many distinct pathological stages associated with complex genetic, epigenetic, molecular, and biological events (9,10). The ultimate goal of advanced molecular analysis of cancer is to provide a comprehensive model that combines molecular and genetic analyses and should aim not only to explain the pathways in the progression of invasive disease, but also to define the relationships between histological variants in a manner that can be translated into practical clinical applications. The simultaneous evaluation of gene expression of multiple genes may prove to be a powerful clinical prognostic or predictive tool (11–13), and these potential applications continue to fascinate and challenge clinical scientists. The accuracy of this microarray data, however, is determined by the specificity/purity of the input RNA. The fundamental advantage of LCM is that it allows separation of cells of interest that may constitute as little as 1% of the volume of the biopsy sample so that molecular analysis can be performed on pure populations. Since its inception, LCM has significantly improved the quality of downstream DNA, RNA, and protein-based studies. This study demonstrates how increased complexity of tissue manipulation affects the integrity of RNA extracted from microdissected cells, with variations in quality up to 3-fold, and defines a rapid (between 50%–80% faster than other methods) protocol that preserves and enhances the histomorphology of fresh frozen breast tissue—facilitating precise dissection of cells with minimal compromise of RNA quality.

We believe that our findings contribute to the current body of literature which strives to optimize the sensitivity of downstream applications of LCM, and that they are helpful to our colleagues working in the same field.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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