

Chapter 2

Laser Microdissection for Gene Expression Profiling

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Abstract

Microarray-based gene expression profiling is revolutionizing biomedical research by allowing expression profiles of thousands of genes to be interrogated in a single experiment. In cancer research, the use of laser microdissection (LM) to isolate RNA from tissues provides the ability to accurately identify molecular profiles from different cell types that comprise the tumor and its surrounding microenvironment. Because RNA is an unstable molecule, the quality of RNA extracted from tissues can be affected by sample preparation and processing. Thus, special protocols have been developed to isolate research-quality RNA after LM. This chapter provides detailed descriptions of protocols used to generate microarray data from high-quality frozen breast tissue specimens, as well as challenges associated with formalin-fixed paraffin-embedded specimens.

Key words: Laser microdissection, Gene expression, Microarray, Frozen tissue, FFPE, Molecular signature, Breast cancer

1. Introduction

Tumorigenesis is a complex process, involving structural changes at multiple chromosomal locations and altered expression of numerous genes and proteins. Early efforts to identify genes involved in cancer development evaluated single genes with known or putative roles in cellular processes such as growth, proliferation, angiogenesis, and apoptosis. While these efforts have resulted in the identification of more than 350 genes (1), additional genes of unknown or presumably unrelated function likely play critical roles in cancer development and progression (2). cDNA microarrays, which allow quantitative, large-scale analysis of gene expression, provide a global approach to identifying

genes involved in tumorigenesis and metastasis without a priori knowledge of the underlying molecular pathways (3). Microarrays have been used to develop molecular signatures that correlate with tumor characteristics or outcomes and are being used in clinical diagnostic tests to guide treatments for patients with breast cancer (4, 5).

Despite the successful development of clinical assays and the publication of hundreds of microarray-based papers, the majority of microarray studies have used RNA isolated from tissue by homogenization or manual microdissection. Because the majority of human tumors are highly heterogeneous, with numerous cell types comprising the primary tumor and surrounding microenvironment, laser microdissection (LM) is necessary to isolate specific cells. For example, RNA isolated from laser-microdissected breast tumor cells will be free from contamination from normal epithelial, stromal, and vascular cells, which could compromise the accuracy of the resulting gene expression profiles.

Because RNA is sensitive to degradation, isolation of RNA after LM requires a defined protocol that includes careful cleaning of all equipment with RNase inhibitors, special histological stains, and rapidity (less than 30 min) in cutting, mounting, and microdissecting the tissues. In this chapter, we present protocols for performing microarray analysis using RNA isolated after LM and describe alternate protocols for gene expression analysis of formalin-fixed paraffin-embedded (FFPE) archival specimens.

2. Materials

2.1. Tissue Sectioning, Staining, and Laser Microdissection

1. Membrane-based laser microdissection slides (W. Nuhsbaum, McHenry, IL).
2. Disposable microtome blades, HP35n, noncoated (Thermo Fisher Scientific, Pittsburgh, PA).
3. 0.5 ml PCR tubes (Eppendorf, Hauppauge, NY).
4. RNaseZap[®] (Applied Biosystems, Carlsbad, CA).
5. Nuclease-free water (Applied Biosystems).
6. LCM Staining Kit (Applied Biosystems) – *store cresyl violet at 4°C*.
7. 50% ethanol.
8. 75% ethanol.
9. 95% ethanol.
10. 100% ethanol.
11. Xylene (used only for FFPE samples).

12. Tissue-Tek® Cryomold® Standard, 25×20×5 mm (Electron Microscopy Sciences, Hatfield, PA).
13. Cryomatrix optimal cutting temperature (OCT) compound (Thermo Fisher Scientific).

2.2. RNA Isolation from Frozen Tissue

1. RNAqueous®-Micro kit (Applied Biosystems).
2. Nuclease-free water.
3. 100% ethanol.
4. Agilent RNA 6000 Pico kit (Agilent Technologies, Santa Clara, CA).
5. Agilent 2100 Bioanalyzer (Agilent Technologies).
6. RNaseZap®.

2.3. Amplification and Fragmentation of RNA from Frozen Tissue

1. MessageAmp™ II aRNA Amplification kit (Applied Biosystems).
2. GeneChip® Eukaryotic Poly-A RNA Control kit (Affymetrix, Santa Clara, CA).
3. 75 mM Bio-11-UTP (Applied Biosystems).
4. Nuclease-free water.
5. 5× Fragmentation buffer, component of the GeneChip® Sample Cleanup Module (Affymetrix).
6. Agilent RNA 6000 Pico kit.
7. Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA).
8. Agilent 2100 Bioanalyzer.
9. NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) – Note: the current model is the NanoDrop 2000.

2.4. Hybridization of aRNA to Microarrays

1. GeneChip® Expression 3' Amplification reagents containing 20× Eukaryotic Hybridization Controls and Control Oligonucleotide B2 (Affymetrix).
2. Herring Sperm DNA (Promega, Madison, WI).
3. Bovine serum albumin (BSA) (Invitrogen, Carlsbad, CA).
4. MES hydrate (Sigma-Aldrich, St Louis, MO).
5. MES sodium salt (Sigma-Aldrich).
6. 5 M NaCl (Sigma-Aldrich).
7. 0.5 M EDTA (Sigma-Aldrich).
8. Tween 20 (Promega).
9. DMSO (Sigma-Aldrich).
10. Nuclease-free water.

11. GeneChip® Human Genome U133A 2.0 Arrays (HG U133A 2.0) (Affymetrix).
12. Hybridization oven (Affymetrix).

2.5. Washing, Staining, and Scanning Microarrays

1. Bovine serum albumin.
2. Streptavidin phycoerythrin (SAPE) (Invitrogen).
3. Goat IgG (Sigma-Aldrich).
4. Biotinylated antistreptavidin (Vector Laboratories, Burlingame, CA).
5. 20× SSPE (Sigma-Aldrich).
6. 5 M NaCl.
7. Tween 20.
8. Nuclease-free water.
9. Tough Spots (T-SPOTS; Diversified Biotech, Boston, MA).
10. Fluidics Station (Affymetrix).
11. Scanner (Affymetrix).

2.6. Quantitative Real-Time Polymerase Chain Reaction of RNA from Frozen Tissue or FFPE

1. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
2. TaqMan® Universal PCR Master Mix (Applied Biosystems).
3. TaqMan® Gene Expression Assays (Applied Biosystems).
4. FirstChoice® Human Brain Reference RNA (Applied Biosystems).
5. iCycler iQ™ PCR plates (Bio-Rad Laboratories, Hercules, CA).
6. iCycler iQ™ thermal seals (Bio-Rad Laboratories).
7. iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories).

2.7. RNA Isolation from Formalin-Fixed Paraffin Embedded Specimens

1. RecoverAll™ total nucleic acid isolation kit (Applied Biosystems).
2. 100% ethanol.

2.8. Commercial Vendor Information

1. Affymetrix – <http://www.affymetrix.com>.
2. Agilent Technologies – <http://www.agilent.com>.
3. Applied Biosystems – <http://www.appliedbiosystems.com>.
4. Bio-Rad Laboratories – <http://www.bio-rad.com>.
5. Diversified Biotech – <http://divbio.com/>.
6. Electron Microscopy Sciences – <http://emsdiasum.com/microscopy/>.
7. Eppendorf – <http://www.eppendorf.com>.

8. Invitrogen – <http://www.invitrogen.com>.
9. Promega – <http://www.promega.com>.
10. Sigma-Aldrich – <http://www.sigmaaldrich.com>.
11. Thermo Fisher Scientific – <http://www.thermofisher.com>.
12. Vector Laboratories – <http://vectorlabs.com/>.
13. W. Nuhsbaum – <http://www.nuhsbaum.com/>.

3. Methods

RNA is extremely susceptible to degradation by RNase enzymes in the environment. To generate high-quality microarray or quantitative real-time polymerase chain reaction (qRT-PCR) data, it is critical to obtain RNA of the highest possible quality by preventing RNase contamination during tissue collection and processing, RNA isolation, and downstream applications. Several general precautions should be taken when working with RNA in the laboratory. All equipment and laboratory benches should be thoroughly cleaned with RNaseZap[®] and then rinsed with nuclease-free or deionized water. All pipette tips, tubes, reagents, and other consumables must be RNase-free. Pipette tips should contain barriers and should be changed each time you pipette, even if you are pipetting the same reagent, to avoid potential cross-contamination between samples and to prevent RNase contamination. For most procedures, it is advisable to use nuclease-free, hydrophobic, nonstick tubes to minimize loss of sample that may otherwise adhere to the tube walls. Gloves should be worn at all times and changed frequently, especially after coming into contact with liquids or surfaces that may be contaminated with RNases.

3.1. Sectioning and Staining

To prevent RNA degradation, tissue sectioning, staining, and LM must be performed as quickly as possible (typically within 30 min). In our laboratory, two individuals perform these steps and process one slide at a time. The LCM Staining Kit employs a novel staining procedure that avoids exposing the tissue sections to pure water at any step, thus minimizing the potential for RNA degradation.

3.1.1. Frozen Tissue

1. In the bottles provided with the LCM Staining Kit, prepare 95, 75, and 50% ethanol solutions by diluting 100% ethanol with nuclease-free water. Add the dehydration beads to the bottle labeled 100% ethanol and add absolute ethanol. Do not use the ethanol in this container to make any of the diluted solutions.

2. Clean the staining containers included in the LCM Staining Kit with RNaseZap[®]. For FFPE samples, a glass staining dish should also be cleaned. Spray the containers generously with RNaseZap[®] and allow them to sit for 10 min. Rinse twice with distilled water and then perform a final rinse with nuclease-free water. Allow the containers to dry under a hood and then fill with the appropriate solutions.
3. Set the temperature of the cryostat to -30°C .
4. Clean the knife holder (*not* the knife blade itself) with 100% ethanol and treat the brushes that will be used to manipulate the tissue sections with RNaseZap[®].
5. Cool the specimen and brushes in the cryostat.
6. Inside the cryostat, remove the frozen OCT-embedded tissue from its cryomold and mount securely to the metal specimen stage with OCT compound, orienting the tissue according to regions of interest (see Note 1).
7. Using a fresh disposable blade, shave OCT from the block until the tissue becomes visible. Set the cutting thickness to 8 μm .
8. Section the tissue and use a small brush to straighten out the newly cut sections.
9. Manipulate sections onto the foil slides (see Note 2).
Perform staining under a hood used only for RNA procedures. Change all containers and blade surfaces between each patient sample.
10. Cut sections at 8 μm and mount onto a membrane-based laser microdissection slide.
11. Wash slide in 95% ethanol for 30 s.
12. Wash in 75% ethanol for 30 s.
13. Wash in 50% ethanol for 30 s (see Note 3).
14. Pipette cresyl violet ($\sim 50\ \mu\text{l}$) onto the slide to completely cover the tissue sections; allow the slide to sit for 15 s.
15. Rinse in 95% ethanol for 5 s.
16. Rinse in 100% ethanol for 5 s.
17. Rinse in a second container of 100% ethanol for 30 s (see Note 4).
18. Allow slide to air dry.

3.1.2. Formalin-Fixed Paraffin-Embedded Tissue

1. Fill the clean staining dish with nuclease-free water and warm on a hot plate to the desired temperature for the paraffin being used (typically $37\text{--}42^{\circ}\text{C}$). Change the water bath between each sample.

2. Cut sections at 8 μm and lay out ribbon onto the warm water bath.
3. Mount sections onto a membrane-based LM slide.
4. Place slides in an incubator set at 56°C for 15 min (see Note 5).
5. Wash in xylene for 1 min; repeat twice for a total of three washes.
6. Wash in 95% ethanol for 30 s.
7. Wash in 75% ethanol for 30 s.
8. Wash in 50% ethanol for 30 s.
9. Pipette Cresyl Violet stain onto the slide using enough volume to cover the sections; allow to sit for 15 s.
10. Rinse in 95% ethanol for 5 s.
11. Rinse in 100% ethanol for 5 s.
12. Rinse in 100% ethanol for 30 s.

3.2. Laser Microdissection

1. Use a cover-slipped H&E section to orient the tissue for microdissection. Estimate the number of cells – in our experience, ~10,000 cells usually yields sufficient RNA for downstream applications.
2. Locate the area on the cresyl violet-stained section to be microdissected (see Note 6).
3. Pipette 60 μl of Lysis solution for OCT-embedded tissues, or 60 μl of digestion buffer for FFPE-embedded tissues, into the cap of a clean 0.5 ml Eppendorf tube. Place the cap into the cap holder apparatus of the laser microdissection system.
4. Microdissect the area of interest (Fig. 1) and drop the sample into the buffer (see Note 7).
5. Add the remaining 40 μl of Lysis solution (OCT tissues) or 340 μl of digestion buffer (FFPE tissues) to the tube and carefully close the lid.

3.3. RNA Isolation from Frozen Tissue

1. Before first use, add 10.5 ml of 100% ethanol to Wash solution 1 and 22.4 ml of 100% ethanol to Wash solution 2/3 and mix well (see Note 8).
2. On first use, thaw the Pico Ladder on ice, centrifuge briefly, and transfer to an RNase-free tube. Heat-denature the ladder for 2 min at 70°C in a heat block, then immediately place on ice. Add 90 μl of nuclease-free water, pipette up and down several times, and flick the tube to mix. Briefly centrifuge the tube and aliquot 5–10 μl to RNase-free tubes. Store at –70°C (see Note 9).

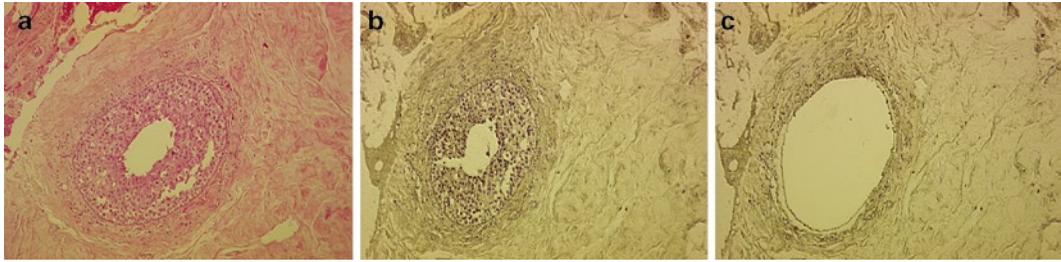


Fig. 1. Staining and laser microdissection of formalin-fixed paraffin-embedded breast tissue containing a ductal carcinoma in situ (DCIS). (a) Standard hematoxylin and eosin (H&E) stain of the DCIS on a glass slide. (b) DCIS on a foil slide stained with Cresyl Violet. (c) Breast tissue after removal of the DCIS by laser microdissection.

3. Place a tube containing nuclease-free water (at least 50 μl per sample) in a heat block at 95°C.
4. Prewarm an air incubator to 42°C.
5. Thaw LCM Additive and 10 \times DNase I buffer on ice.
6. Flick the tube containing the microdissected sample several times and centrifuge briefly. Place the sample in the 42°C incubator for 30 min (see Note 10).
7. Approximately 6–7 min prior to completion of the 30-min incubation, prewet the Micro Filter by adding 30 μl of Lysis solution to the filter, which is placed in a Micro-Elution Tube (Micro Filter Cartridge Assembly). After 5 min, centrifuge the Micro Filter Cartridge Assembly for 30 s at 16,000 rcf to remove the Lysis solution from the filter.
8. Remove the microdissected sample from the 42°C incubator, vortex on maximum speed by pulsing three times, and centrifuge briefly. Add 3 μl of LCM Additive, mix by vortexing, and centrifuge briefly.
9. Add 52 μl of 100% ethanol and mix completely into the sample by pipetting up and down (see Note 11). Transfer the sample to the center of the filter in the Micro Filter Cartridge Assembly. Centrifuge for 1 min at 10,000 rcf (see Note 12).
10. Add 180 μl of Wash solution 1 to the filter and centrifuge for 1 min at 10,000 rcf.
11. Add 180 μl of Wash solution 2/3 and centrifuge for 30 s at 16,000 rcf. Repeat this step one time.
12. Remove the filter from the collection tube and discard the flow-through. Recap the assembly and centrifuge for 1 min to remove trace amounts of liquid.
13. Remove the filter containing the sample and place in a new Micro Elution Tube.
14. Add 10 μl of nuclease-free water heated to 95°C in step 1 above to the center of the filter (see Note 13). Incubate the

- assembly for 5 min at room temperature, then centrifuge for 1 min at 16,000 rcf to elute the RNA. Repeat this step with a second 10 μ l volume of 95°C nuclease-free water, incubate, and centrifuge.
15. Remove the filter and place the sample on ice.
 16. Add 2 μ l of 10 \times DNase I buffer and 1 μ l of DNase I to the sample and mix by gently flicking the tube. Centrifuge briefly and incubate for 20 min in a heat block at 37°C. During the incubation, remove the DNase Inactivation Reagent from the freezer and thaw at room temperature.
 17. Remove the sample from the heat block. Vigorously vortex the DNase Inactivation Reagent and add 2.3 μ l to the sample. Gently tap the side of the tube to mix and incubate for 2 min at room temperature. After 1 min, vortex the sample, tap the tube to move all contents to the bottom, and continue the incubation for 1 min.
 18. Centrifuge the sample for 1 min 30 s at 16,000 rcf to pellet the DNase Inactivation Reagent. Transfer the supernatant containing the RNA to a new tube without disturbing the pellet, then place the RNA on ice (see Note 14).

3.4. Assessing RNA Integrity

1. Remove an aliquot of the Pico Ladder from the freezer and thaw on ice. Remove the Pico Gel Matrix, Pico Dye Concentrate, Pico Conditioning Solution, and Pico Marker from 4°C and allow the reagents to warm to room temperature for at least 30 min. Ensure that the Dye Concentrate is shielded from light (see Note 15).
2. Add 550 μ l of Gel Matrix to a Spin Filter and centrifuge for 10 min at 1,500 rcf. Aliquot 65 μ l of filtered gel into the tubes provided with the kit (produces seven to eight tubes of filtered gel). The filtered gel may be stored at 4°C for up to 2 months.
3. Vortex the tube of Dye Concentrate for 10 s and then centrifuge briefly. Add 1 μ l of Dye Concentrate to a tube of filtered gel (warmed to room temperature), vortex for 10 s, then centrifuge for 10 min at 16,000 rcf. One tube of gel-dye mix can be used to run two chips per day.
4. Transfer 1.25–1.5 μ l of each RNA sample into a 0.65-ml tube. Heat the sample for 2 min in a heat block at 65–70°C. Place on ice for ~5 min to cool, then centrifuge briefly to collect the RNA at the bottom of the tube.
5. Start the 2100 Expert Software and turn on the Bioanalyzer. Place an electrode cleaner containing 350 μ l of nuclease-free water in the instrument and close the lid (see Note 16). On the instrument menu, select “Assays,” “Electrophoresis,”

“RNA,” and finally “Eukaryotic Total RNA Pico Series.” Select the number of samples (from 1 to 11) to be assayed. Enter the sample information and any additional comments pertaining to that sample.

6. Place the Pico Chip on the chip priming station (ensuring that the base plate is on “C”) and pull the syringe back to 1 ml. Add 9 μ l of gel-dye mix to the well labeled with an encircled “G” (see Note 17). Close the chip priming station until you hear a click, then press the syringe down until it is secured beneath the syringe clip. After 30 s, release the clip, wait 5 s, and pull the syringe back to the 1 ml mark.
7. Add 9 μ l of gel-dye mix to the two remaining wells marked “G.” Add 9 μ l of Pico Conditioning Solution to the well marked “CS.” Add 5 μ l of Pico Marker to the ladder well and to each well that will contain an RNA sample. Add 6 μ l of Pico Marker to any empty sample wells.
8. Add 1 μ l of diluted Pico Ladder to the ladder well and 1 μ l of sample to the appropriate sample well. After loading all wells, vortex the chip using the manufacturer-supplied vortex for 1 min at 2,400 rpm. During this time, remove the electrode cleaner from the instrument. Place the Pico Chip on the Agilent 2100 Bioanalyzer and begin the run by pressing “Start” (see Notes 18 and 19) (Fig. 2).

3.5. Amplification of RNA from Frozen Tissue

When using small amounts of RNA for gene expression analysis, it is often necessary to first amplify the RNA to generate sufficient material for hybridization to the microarray. For RNA isolated

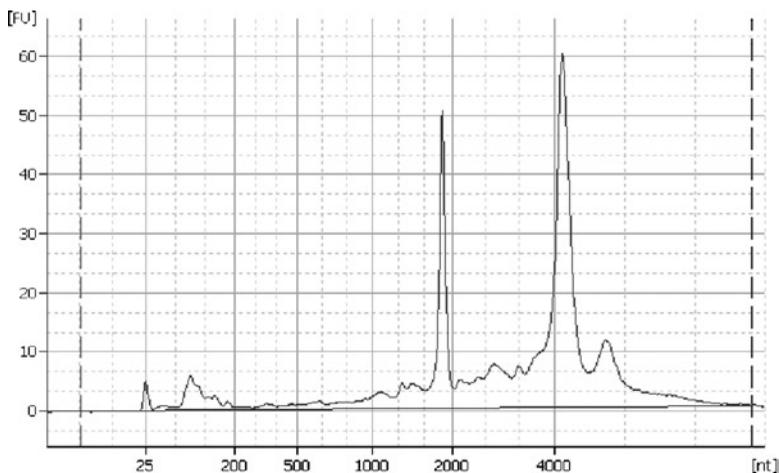


Fig. 2. Electropherogram of total RNA isolated from frozen breast tissue collected via laser microdissection using the RNAqueous[®]-Micro kit. The RNA (RIN = 8.7) was assayed on the Bioanalyzer using a Pico Chip. The 18S rRNA and 28S rRNA peaks are visible near 2,000 and 4,000 nucleotides (nt), respectively.

from laser-microdissected tissues, two rounds of amplification are normally required. All frozen reagents for the amplification protocol should be thawed on ice; enzymes should be stored at -20°C immediately prior to and after use. All master mixes should be prepared in excess (generally $\sim 5\%$) to avoid running short of master mix when working with large numbers of samples.

1. Completely thaw the Poly-A Control Stock on ice, then add $2\ \mu\text{l}$ to a small tube. Add $38\ \mu\text{l}$ of nuclease-free water and mix well by vortexing or flicking the tube. Centrifuge briefly to collect the liquid at the bottom of the tube. This is the first dilution and can be stored at -80°C for up to 6 weeks (or eight freeze-thaw cycles).
2. Remove $2\ \mu\text{l}$ of the first dilution and place in a new tube. Add $98\ \mu\text{l}$ of nuclease-free water to make the second dilution. Mix well and centrifuge briefly.
3. Combine $2\ \mu\text{l}$ of the second dilution with $98\ \mu\text{l}$ of nuclease-free water to make the third dilution. Mix well and centrifuge.
4. Combine $2\ \mu\text{l}$ of the third dilution with $18\ \mu\text{l}$ of nuclease-free water to prepare the fourth dilution. Mix well and centrifuge.
5. Combine $2\ \mu\text{l}$ of the fourth dilution with $18\ \mu\text{l}$ of nuclease-free water to prepare the fifth dilution. Mix well and centrifuge (see Note 20).
6. Using the estimated RNA concentration obtained from the Bioanalyzer, calculate the volume of sample containing 10 ng of RNA (see Note 21). Transfer this volume to a 0.2 ml PCR tube and adjust the total volume to $9\ \mu\text{l}$ with nuclease-free water. If the volume needed for 10 ng of RNA is greater than $9\ \mu\text{l}$, transfer this amount to a hydrophobic, nonstick microcentrifuge tube, and centrifuge in a vacuum concentrator until the volume is $\leq 9\ \mu\text{l}$. Transfer the concentrated sample to a 0.2-ml PCR tube and adjust the volume to $9\ \mu\text{l}$ with nuclease-free water.
7. Flick the tubes to mix and centrifuge briefly to collect the liquid at the bottom of the tube.

3.6. First Round Amplification

1. Add $2\ \mu\text{l}$ of the fifth dilution of the Poly-A Controls to each sample containing 10 ng of RNA (see Note 22). Flick the tubes to mix and centrifuge briefly.
2. Add $1\ \mu\text{l}$ of Oligo(dT) primer to each sample, flick the tubes to mix, and centrifuge briefly. Incubate samples for 10 min at 70°C in a thermal cycler.
3. Remove samples from the thermal cycler, centrifuge briefly, and place on ice.

4. In a small tube, prepare a master mix containing the following for each sample:
 - (a) 2 μ l 10 \times first strand buffer.
 - (b) 4 μ l dNTP mix.
 - (c) 1 μ l RNase inhibitor.
 - (d) 1 μ l ArrayScript™.

Vortex the tube to mix and centrifuge briefly to collect the contents at the bottom of the tube. Add 8 μ l of the master mix to each sample, flick the tubes to mix, and centrifuge. Incubate samples for 2 h at 42°C in an air incubator or hybridization oven, then centrifuge briefly, and place on ice.

5. Prepare a master mix on ice containing the following reagents for each sample:
 - (a) 63 μ l nuclease-free water.
 - (b) 10 μ l 10 \times second strand buffer.
 - (c) 4 μ l dNTP mix.
 - (d) 2 μ l DNA polymerase.
 - (e) 1 μ l of RNase H.

Vortex to mix and centrifuge briefly to collect the master mix at the bottom of the tube. Add 80 μ l of master mix to each sample, flick the samples to mix, and centrifuge briefly. Incubate the samples in a precooled thermal cycler for 2 h at 16°C (see Note 23), then centrifuge briefly and place on ice.

6. Place a tube containing at least 30 μ l of nuclease-free water per sample in a heat block set to 50–55 °C. For each sample, place a filter inside a cDNA Elution tube. Note: add 24 ml of 100% ethanol to the Wash buffer before using for the first time.
7. Transfer the samples from the 0.2 ml tubes to 1.5 ml microcentrifuge tubes. Add 250 μ l of cDNA Binding buffer to each sample, mix by pipetting up and down and then flicking the tubes several times. Centrifuge samples briefly, then transfer each sample to the filter of a cDNA Filter Cartridge. Centrifuge samples for 1 min at 10,000 rcf, then discard the flow-through.
8. Add 500 μ l of Wash buffer to each filter. Centrifuge for 1 min at 10,000 rcf and discard the flow-through.
9. Centrifuge the cDNA Filter Cartridges for 1 min at 10,000 rcf to remove any residual liquid from the filter. Transfer filters to new cDNA Elution tubes and discard the old tubes.
10. Add 10 μ l of nuclease-free water warmed to 50–55°C to the center of each filter. Incubate for 2 min at room temperature. Elute samples by centrifuging for 1 min 30 s at 10,000 rcf. Repeat this step using a second 10 μ l volume of warm nuclease-free water.

11. Discard filters and place tubes containing the eluted cDNA on ice.
12. Prepare the in vitro transcription (IVT) master mix at room temperature. Note that for the first round of amplification, the IVT reactions contain only unmodified dNTPs. For each sample include:
 - (a) 4 μ l T7 ATP.
 - (b) 4 μ l T7 CTP.
 - (c) 4 μ l T7 GTP.
 - (d) 4 μ l T7 UTP.
 - (e) 4 μ l T7 10 \times reaction buffer.
 - (f) 4 μ l T7 enzyme mix.

Vortex the master mix and centrifuge briefly to collect the contents at the bottom of the tube. Aliquot 24 μ l of master mix to each sample, flick the tubes to mix, and centrifuge briefly. Incubate samples for 14 h in an air incubator or hybridization oven at 37°C.

13. Place a tube containing nuclease-free water in a heat block at 50–55°C – we recommend heating at least 120 μ l of nuclease-free water per sample.
14. For each sample, place an aRNA Filter Cartridge in an aRNA Collection Tube.
15. Remove the IVT reactions from the incubator. Add 60 μ l of nuclease-free water to each sample, mix by flicking the tube, and centrifuge briefly. Add 350 μ l of aRNA Binding buffer followed by 250 μ l of 100% ethanol to each sample. Mix the samples by pipetting up and down at least five times, then transfer each sample to an aRNA Filter Cartridge. Centrifuge samples for 1 min at 10,000 rcf, then discard the flow-through and remount the filter on the collection tube.
16. Add 650 μ l of wash buffer to each Filter Cartridge and centrifuge for 1 min at 10,000 rcf. Discard the flow-through and place the Filter Cartridge back inside the collection tube. Centrifuge samples for an additional 1 min at 10,000 rcf to remove residual wash buffer. Discard the flow-through and place the Filter Cartridge in a new collection tube.
17. Apply 100 μ l of nuclease-free water warmed to 50–55°C to the center of each filter. Incubate at room temperature for 2 min, then centrifuge for 1 min at 10,000 rcf to elute the aRNA.
18. Remove 3 μ l of the aRNA and transfer to a small tube. Heat the samples for 2 min in a heat block at 65–70°C. Place samples on ice to cool, then centrifuge the samples briefly, and return to ice.

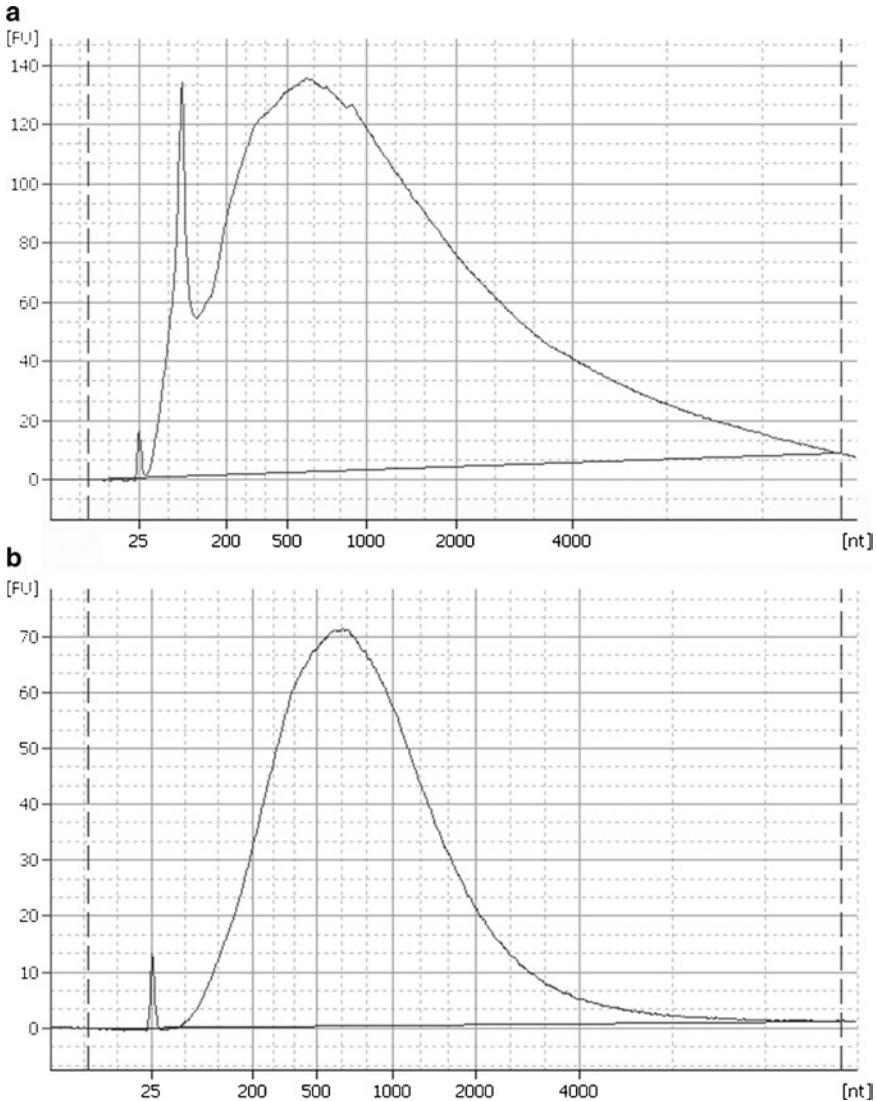


Fig. 3. Electropherograms of RNA isolated from frozen breast tissue following one and two rounds of amplification. (a) Total RNA amplified using the MessageAmp™ II aRNA Amplification kit and assayed on the Bioanalyzer using a Pico Chip. (b) Second round aRNA assayed using a Nano Chip. The majority of the second round aRNA product should be >500 nucleotides (nt) in length.

19. Run 1 μ l of the first round aRNA samples from step 18 on the Bioanalyzer using a Pico Chip following the instructions outlined above (Fig. 3a). Use 1.5 μ l of the remaining aRNA to measure the concentration of each sample on a NanoDrop ND-1000 Spectrophotometer.

3.7. Second Round Amplification

1. Calculate the volume of first round aRNA (using the concentration obtained on the NanoDrop) needed to obtain 1 μ g of starting material for the second round of amplification

(see Note 24). If this volume exceeds 10 μl for any sample, concentrate those samples in a vacuum concentrator to less than 10 μl . In a 0.2-ml PCR tube, adjust the volume of all samples to 10 μl using nuclease-free water.

2. Add 2 μl of second round primers to each aRNA sample. Flick the tubes to mix and centrifuge briefly. Place samples in a thermal cycler heated to 70°C for 10 min, then centrifuge briefly and place on ice.
3. Prepare a master mix containing the following for each sample:
 - (a) 2 μl 10 \times first strand buffer.
 - (b) 4 μl dNTP mix.
 - (c) 1 μl RNase inhibitor.
 - (d) 1 μl ArrayScript™.

Vortex the master mix and centrifuge briefly. Add 8 μl of master mix to each sample and flick the tubes to mix. Centrifuge briefly and incubate for 2 h at 42°C in an air incubator or hybridization oven.

4. Following incubation, centrifuge the samples briefly and place on ice. Add 1 μl of RNase H to each sample, flick the tubes to mix, and centrifuge briefly to collect the contents at the bottom of the tube. Incubate samples for 30 min at 37°C in an air incubator or hybridization oven, then centrifuge briefly and place on ice.
5. Add 5 μl of the Oligo(dT) primer to each sample, flick the tubes to mix, and centrifuge briefly. Incubate samples for 10 min at 70°C in a thermal cycler, then centrifuge and place on ice.
6. Prepare a master mix on ice for the second strand synthesis that includes the following for each sample:
 - (a) 58 μl nuclease-free water.
 - (b) 10 μl 10 \times second strand buffer.
 - (c) 4 μl dNTP mix.
 - (d) 2 μl DNA polymerase.Vortex to mix and add 74 μl to each sample. Flick the tubes to mix and centrifuge briefly. Incubate samples for 2 h in a thermal cycler that has been precooled to 16°C. Remove samples from the thermal cycler, centrifuge briefly and place on ice.
7. Purify the cDNA following the exact procedure outlined above.

8. Prepare a master mix at room temperature that contains for each sample:
 - (a) 4 μl T7 ATP.
 - (b) 4 μl T7 CTP.
 - (c) 4 μl T7 GTP.
 - (d) 2.6 μl T7 UTP.
 - (e) 1.4 μl biotin-11-UTP.
 - (f) 4 μl T7 reaction buffer.
 - (g) 4 μl T7 enzyme mix.

Vortex and centrifuge briefly to collect the contents at the bottom of the tube. Add 24 μl of master mix to each sample, flick the tubes to mix, and centrifuge briefly. Incubate the samples for 14 h at 37°C in an air incubator or hybridization oven.

9. Purify the second round, labeled aRNA using the same procedure for aRNA purification outlined above.
10. Run the second round, labeled aRNA on the Bioanalyzer using the Agilent RNA 6000 Nano kit. Reagent and sample preparation for the Nano Chip is very similar to that for the Pico Chip with minor exceptions. Warm all refrigerated Nano reagents to room temperature. Prepare the filtered gel and gel-dye mix using the Nano Gel Matrix and Nano Dye Concentrate as outlined above.
11. Thaw the Nano Ladder on ice. Flick the tube several times and centrifuge briefly. Transfer 2.5 μl of the Nano Ladder to a new tube. Prepare 5–10 μl aliquots of the remaining ladder and store at –20°C for future use. Place 3 μl of each aRNA sample in a small tube. Heat the samples and ladder for 2 min in a heat block at 65–70°C. Place the samples on ice to cool, then centrifuge briefly to collect any condensation at the bottom of the tube.
12. Start the 2100 Expert Software and clean the electrodes as previously described. Select the “Eukaryotic Total RNA Nano Series Assay” and select the number of samples (from 1 to 12) that will be run. Enter the sample information.
13. Load the Nano Chip using the same procedure for loading the Pico Chip, but note that the Nano Chip does not use Conditioning Solution, allowing 12 samples to be run (Fig. 3b).
14. Measure the concentration of 1.5 μl of the remaining second round aRNA on the NanoDrop.

3.8. Fragmentation of Labeled aRNA

1. For each sample, transfer 15 μg of labeled aRNA to a 0.2-ml PCR tube. The aRNA yield after two rounds of amplification typically exceeds 1,500 ng/ μl and often is ~2,000 ng/ μl ; therefore, you should not have to vacuum-concentrate the samples prior to fragmentation. Add nuclease-free water to

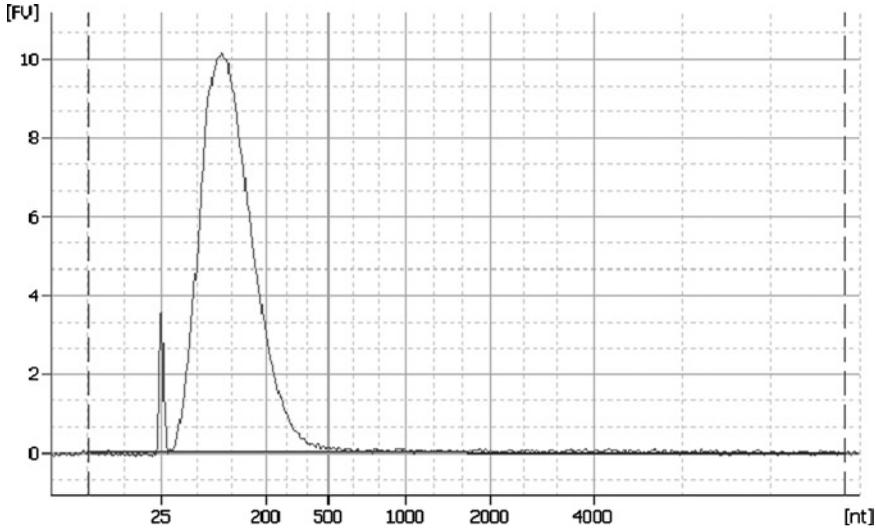


Fig. 4. Electropherogram of fragmented, second round aRNA assayed on the Bioanalyzer using a Nano Chip. The aRNA fragments should be ~35 to 200 nucleotides (nt) in length.

- each sample to bring the total volume to 24 μ l. Flick the tubes to mix and centrifuge briefly.
2. Add 6 μ l of 5 \times Fragmentation buffer to each sample, flick the tubes to mix, and briefly centrifuge. Fragment the aRNA samples by incubating for 35 min at 94°C in a thermal cycler. Place the samples on ice to cool, then centrifuge briefly to collect condensation at the bottom of the tube.
3. Run the fragmented samples on a Bioanalyzer Nano Chip (see Note 25) (Fig. 4).

3.9. Hybridization of Fragmented aRNA to Microarrays

Microarrays for gene expression analysis are available from several commercial vendors, including Affymetrix, Agilent Technologies, and Illumina. In this chapter, we provide protocols for hybridization of labeled, fragmented aRNA to the Affymetrix HG U133A 2.0 arrays. The protocol is the same for all eukaryotic arrays manufactured by Affymetrix; however, the starting amount of fragmented aRNA and volume of reagents used in certain steps will vary across the different array formats.

1. Thaw the 20 \times Eukaryotic Hybridization Controls on ice. Mix well by flicking the tube or vortexing gently and then centrifuge briefly to collect the contents at the bottom of the tube. Prepare aliquots containing the volume used during a typical hybridization set-up (we usually hybridize eight samples at a time) and store at -20°C.
2. Prepare 1 l of 12 \times MES stock buffer by dissolving 64.61 g of MES hydrate and 193.3 g of MES sodium salt in 800 ml of nuclease-free water. When completely dissolved, adjust

the volume to 1,000 ml with nuclease-free water and filter through a 0.22- μ m filter. The pH should be between 6.5 and 6.7. Store at 4°C wrapped in foil to shield from light. Discard the solution if it turns yellow.

3. Prepare 50 ml of 2 \times hybridization buffer by combining:

- (a) 8.3 ml 12 \times MES stock buffer.
- (b) 17.7 ml 5 M NaCl.
- (c) 4.0 ml 0.5 M EDTA.
- (d) 19.9 ml nuclease-free water.
- (e) 100 μ l 10% Tween 20.

Filter the solution through a 0.22- μ m filter and store wrapped in foil at 4°C.

4. Approximately 1 h before setting up the hybridization, remove the HG U133A 2.0 arrays from the refrigerator and allow them to equilibrate to room temperature. Thaw the Control Oligonucleotide B2, 20 \times Eukaryotic Hybridization Controls, Herring Sperm DNA, and BSA on ice. Set the temperature of the hybridization oven to 45°C.

5. Transfer 20 μ l (10 μ g) of fragmented aRNA to a 1.5-ml microcentrifuge tube.

6. Prepare a 1 \times solution of hybridization buffer by combining equal volumes of 2 \times hybridization buffer and nuclease-free water; vortex to mix well.

7. Heat the 20 \times Eukaryotic Hybridization Controls for 5 min at 65°C in a heat block before adding to the hybridization master mix.

8. Prepare the hybridization master mix (at 5% excess) by combining for each sample:

- (a) 3.3 μ l Control Oligonucleotide B2.
- (b) 10 μ l 20 \times Eukaryotic Hybridization Controls.
- (c) 2 μ l Herring Sperm DNA.
- (d) 2 μ l BSA.
- (e) 100 μ l 2 \times hybridization buffer.
- (f) 20 μ l DMSO.
- (g) 42.7 μ l nuclease-free water.

Vortex to mix well, centrifuge briefly, and add 180 μ l of the master mix to each fragmented sample (from step 2 above). Vortex the samples, centrifuge briefly, and incubate for 5 min at 99°C in a heat block.

9. Place the arrays face down on a laboratory tissue. Insert a pipette tip into the top right septum on the back of the array to permit air to vent when filling the array. Fill the arrays with 160 μ l of 1 \times hybridization buffer, remove the pipette tip vent,

and incubate the arrays for 10 min at 60 rpm in the hybridization oven set to 45°C.

10. Transfer the samples from 99°C to a 45°C heat block. Incubate for 5 min, then centrifuge the samples for 5 min at 16,000 rcf.
11. Remove the arrays from the hybridization oven. Insert the pipette tip vent and remove the hybridization buffer. Load 130 µl of the sample into the array, making sure that a bubble is present that can freely move when the array is slowly tilted from side to side. Incubate the arrays for 16 h at 45°C in the hybridization oven while rotating at 60 rpm (see Notes 26 and 27).

3.10. Washing, Staining, and Scanning of Microarrays

1. Prepare 250 ml of 2× stain buffer by combining:

- (a) 41.7 ml 12× MES stock buffer.
- (b) 92.5 ml 5 M NaCl.
- (c) 2.5 ml 10% Tween 20.
- (d) 113.3 ml nuclease-free water.

Filter through a 0.22-µm filter, wrap in foil to shield from light, and store at 4°C.

2. Reconstitute the goat IgG by adding 150 mM NaCl to make a 10 mg/ml solution. For example, add 1 ml of 150 mM NaCl to 10 mg of lyophilized goat IgG. Aliquots should be stored at -20°C, but once thawed for use, store at 4°C.

3. Add 1 ml of nuclease-free water to reconstitute the biotinylated antistreptavidin antibody to 0.5 mg/ml. Gently pipette up and down to mix, then store at 4°C.

4. Prepare 1 l of Wash A solution by combining:

- (a) 300 ml 20× SSPE.
- (b) 699 ml deionized water.
- (c) 1 ml 10% Tween 20.

Filter through a 0.22-µm filter and store at room temperature.

5. Prepare 1 l of Wash B solution by combining:

- (a) 83.3 ml 12× MES stock buffer.
- (b) 5.2 ml 5 M NaCl.
- (c) 1 ml 10% Tween 20.
- (d) 910.5 ml deionized water.

Filter through a 0.22-µm filter, cover with foil, and store at 4°C.

6. Prepare a master mix (in 5% excess) of the SAPE Solution (stains 1 and 3) by combining for each sample:

- (a) 600 µl 2× stain buffer.
- (b) 48 µl BSA.
- (c) 12 µl SAPE (vortex well before pipetting).

(d) 540 μ l of deionized water.

Vortex to mix. For each sample, aliquot 600 μ l of the SAPE Solution into two 1.5 ml microcentrifuge tubes.

7. Prepare a master mix for the Antibody Solution (Stain 2) in 5% excess. For each sample, combine:
 - (a) 300 μ l 2 \times stain buffer.
 - (b) 24 μ l BSA.
 - (c) 6 μ l IgG.
 - (d) 3.6 μ l biotinylated antistreptavidin.
 - (e) 266.4 μ l deionized water.

Mix well by vortexing and aliquot 600 μ l of the Antibody Solution master mix into a 1.5-ml microcentrifuge tube for each sample.

8. After 16 h, remove the arrays from the hybridization oven. Insert the pipette tip vent and remove the sample solution from the arrays. Fill the arrays with 160 μ l of Wash A solution.
9. Start the GeneChip[®] Operating System (GCOS) or Affymetrix[®] GeneChip[®] Command Console[®] (AGCC) and enter the sample and experiment information.
10. Replace the appropriate water bottles on the fluidics station with Wash A solution and Wash B solution. Prime all modules on the fluidics station.
11. Select the appropriate wash and stain protocol for your arrays and press “Run.” Load the arrays and staining tubes onto the fluidics station. The protocol will automatically run until staining of the arrays is complete (usually ~1 h and 15 min).
12. Remove the arrays from the fluidics station. Place Tough Spots over the septa on the back of the arrays to prevent leakage during scanning. Once the scanner has warmed up (10–15 min), scan the arrays using the GCOS or AGCC software. When scanning is complete, zoom in on each scanned image and check the entire image for any abnormalities. The scanned images can now be analyzed to generate signal intensities for the probes on the arrays as well as a QC report.
13. Once the wash and stain protocol is complete, replace the Wash A solution and Wash B solution with deionized water. Run the Shutdown protocol on all modules, and when finished, turn off the fluidics station.

**3.11. Quantitative
Real-Time Polymerase
Chain Reaction Assays
Using RNA from
Frozen Tissue**

Gene expression differences identified by microarray analysis are frequently confirmed using qRT-PCR. Due to the low yield of RNA following laser microdissection of small tissue sections, RNA is amplified for a single round prior to performing qRT-PCR. To amplify the RNA, follow the procedure detailed above; however, it is not necessary to add the Poly-A controls.

Note that there are several different chemistries available for performing qRT-PCR, including TaqMan® probes, Molecular Beacons, Scorpions®, or SYBR® Green. The following protocol uses TaqMan® Gene Expression Assays, which utilize the same PCR conditions so there is no need to design PCR primers or optimize PCR conditions. In order to achieve accurate results from qRT-PCR, be careful and precise when performing each step in the protocol, especially pipetting.

1. Reverse transcribe the aRNA (one round of amplification) using the High-Capacity cDNA Reverse Transcription Kit. Up to 2 µg of RNA (or aRNA) can be reverse transcribed (see Note 28). In addition, reverse transcribe an appropriate amount of FirstChoice® Human Brain Reference RNA or other reference RNA which will be used to calibrate the relative levels of gene expression in the samples of interest.
2. If necessary, vacuum concentrate the samples to ≤10 µl and increase the volume to 10 µl with nuclease-free water in a 0.2-ml PCR tube.
3. Prepare the reverse transcription master mix (in 5% excess) containing the following for each sample:
 - (a) 2 µl 10× RT buffer.
 - (b) 0.8 µl 25× dNTP mix.
 - (c) 2 µl 10× RT random primers.
 - (d) 1 µl MultiScribe Reverse Transcriptase.
 - (e) 1 µl RNase inhibitor.
 - (f) 3.2 µl nuclease-free water.Vortex to mix, centrifuge briefly, and add 10 µl of the master mix to each sample.
4. Flick the sample tubes to mix and centrifuge briefly to collect the contents at the bottom of the tube. Incubate the samples in a thermal cycler using the following program: 10 min at 25°C, 2 h at 37°C, 5 s at 85°C, and then hold at 4°C.
5. When the reverse transcription reaction is complete, add nuclease-free water to adjust the concentration of the samples and the reference to 5 ng/µl. For example, if 1 µg of aRNA was reverse transcribed in a 20-µl reaction, add 180 µl of water to bring the concentration to 1 µg/200 µl or 5 ng/µl.
6. Mix the diluted samples well and centrifuge briefly. Place on ice or store at -20°C.
7. Perform the qRT-PCR using the TaqMan® Gene Expression Assay of interest (see Note 29). These assays can be run on a variety of real-time instruments, including the Bio-Rad iCycler™. Turn the iCycler™ on and allow it to warm up for at

least 15 min. Enter the plate set-up sample information, select and load the appropriate fluorophore (FAM-490), and save the file.

8. qRT-PCR reactions should be performed in duplicate, but if sufficient starting material is available, triplicate or quadruplicate reactions are recommended. The following protocol is based on 10 ng of cDNA per reaction (see Note 30). Pipette 2 μ l of cDNA (5 ng/ μ l) into the appropriate well of an iCycler™ PCR plate.
9. Prepare a master mix containing the following per reaction:
 - (a) 2.5 μ l TaqMan® Gene Expression Assay (20 \times).
 - (b) 20.5 μ l nuclease-free water.
 - (c) 25 μ l TaqMan® Universal PCR Master Mix.

When running a full 96-well plate, make a master mix for 100 reactions (excess of four reactions). Vortex the master mix and centrifuge briefly to bring the contents to the bottom of the tube. Add 48 μ l of master mix to each sample well. Place a thermal seal on the plate, centrifuge the plate for ~10 s at ~3,000 rcf, and then load the plate on the iCycler™. Set the reaction volume to 50 μ l and run the following protocol: 95°C for 10 min, 50 cycles of 95°C for 15 s followed by 60°C for 1 min, then hold at 4°C.

10. Average the C_t values for each sample and the reference (see Note 31). Calculate the relative transcript levels for each sample using the Comparative C_t Method (see Note 32).

3.12. RNA Isolation from Formalin-Fixed Paraffin-Embedded Specimens

Archived FFPE tissues represent a valuable source of molecular information for the study of cancer (6–8). Although formalin fixation and paraffin embedding preserves tissue structure and cellular morphology, protein–protein and protein–nucleic acid cross links form during the preservation process, which chemically modify and damage the nucleic acids. RNA is particularly susceptible to fragmentation by FFPE, often making RNA isolated from FFPE tissues unusable for molecular analysis. The following protocol is specifically designed to recover RNA from FFPE samples that can be used for downstream applications such as qRT-PCR.

1. Add 42 ml of 100% ethanol to the Wash 1 concentrate and 48 ml of 100% ethanol to the Wash 2/3 concentrate. Mix well.
2. Place the laser microdissected FFPE samples in 400 μ l of digestion buffer. Add 4 μ l of protease to each sample and mix the tubes gently ensuring that the samples are entirely immersed in buffer. Incubate the samples in a heat block set to 50°C for 3 h (see Note 33).

3. Add 480 μ l of isolation additive to each sample and vortex to mix. The solution should be white and cloudy (see Note 33).
4. Add 1.1 ml of 100% ethanol and mix by carefully pipetting the sample up and down. The sample should become clear after mixing (see Note 33).
5. Place a filter cartridge inside a collection tube provided with the kit. Apply 700 μ l of the sample to the filter, centrifuge for 1 min at 10,000 rcf, then discard the flow-through and return the filter to the same collection tube.
6. Apply an additional 700 μ l of sample to the filter and centrifuge for 1 min at 10,000 rcf. Continue this process until the entire sample has been passed through the filter.
7. Add 700 μ l of Wash 1 solution to the Filter Cartridge and centrifuge for 30 s at 10,000 rcf. Discard the flow-through.
8. Add 500 μ l of Wash 2/3 solution to the filter and centrifuge for 30 s at 10,000 rcf. Discard the flow-through. Centrifuge the Filter Cartridge assembly an additional 30 s at 10,000 rcf to remove residual liquid from the filter.
9. Treat the samples in the filters with DNase by combining for each sample:
 - (a) 6 μ l 10 \times DNase buffer.
 - (b) 4 μ l DNase.
 - (c) 50 μ l nuclease-free water.Add this solution to the center of each filter and incubate for 30 min at room temperature.
10. Add 700 μ l of Wash 1 to the filters, incubate for 1 min at room temperature, then centrifuge for 30 s at 10,000 rcf. Discard the flow-through.
11. Add 500 μ l of Wash 2/3 to the filters and centrifuge for 30 s at 10,000 rcf. Discard the flow-through. Add an additional 500 μ l of Wash 2/3 to the filters, centrifuge for 1 min at 10,000 rcf, then transfer the filters to new collection tubes.
12. Add 30 μ l of nuclease-free water heated to 95°C to the center of each filter (see Note 33). Incubate at room temperature for 1 min and then centrifuge for 1 min at 16,000 rcf. Repeat this step using a second 30 μ l volume of heated nuclease-free water.
13. Read the concentration of the eluted samples on the NanoDrop and run the samples on a Bioanalyzer Pico Chip as described in Subheading 3.2.4 (Fig. 5).
14. Store samples at -80°C.

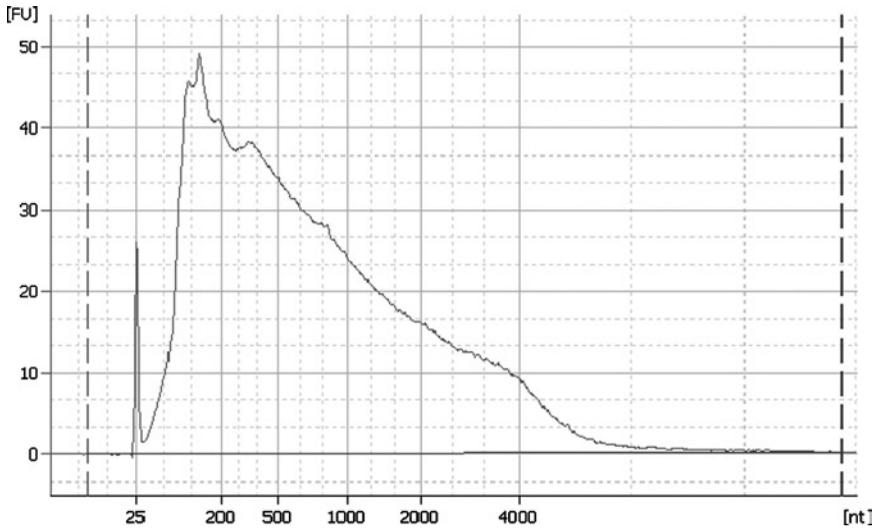


Fig. 5. Electropherogram of total RNA isolated from formalin-fixed paraffin-embedded breast tissue following laser microdissection. RNA was isolated using the RecoverAll™ Total Nucleic Acid Isolation Kit and assayed on the Bioanalyzer using a Pico Chip. Note that the 18S rRNA and 28S rRNA are completely degraded and thus not distinguishable.

3.13. Quantitative Real-Time Polymerase Chain Reaction Assays Using RNA from FFPE

RNA extracted from FFPE tissues is usually not conducive to amplification using an oligo(dT) primer due to fragmentation and degradation. Therefore, qRT-PCR is performed directly on RNA from FFPE tissues – there is no RNA amplification step.

1. Reverse transcribe the FFPE RNA as described.
2. Following reverse transcription of the FFPE RNA to cDNA, perform qRT-PCR as outlined for frozen tissue, but increase the number of PCR cycles to 60 cycles.
3. Average the C_t values for each sample and the reference as described above. Calculate the relative transcript levels for each sample using the Comparative C_t Method.

4. Notes

1. Flash-frozen tissues can only be sectioned after embedding in OCT. To embed a flash-frozen tissue sample in OCT, place the tissue and plastic cryomold in the cryostat set to -30°C . Cover the bottom of the cryomold with OCT, orient the tissue so that the region of interest is facing up, then fill the mold with additional OCT. Allow the compound to solidify – the block will turn white in color when completely frozen.
2. The foil slides should be at room temperature before applying a tissue section. Tissues will not properly adhere to cold slides. Note: If you decide to place more than one tissue section on

a slide to maximize resources, keep the slide in the cryostat while placing the additional tissue section(s) onto the slide. If the additional section does not adhere to the cold slide, warm the area of the slide where the tissue will be deposited by pressing your thumb to the reverse side. Work as quickly as possible.

3. If the OCT is not completely dissolved after the 50% ethanol wash, make sure that the solution is mixed properly. Shake the original container, add fresh solution, and wash the slide again for 30 s.
4. When staining multiple slides per patient sample, change the second 100% ethanol solution when it is noticeably purple in color, indicating that it is no longer a 100% solution. A 100% solution prevents leaching of stain from the slide and allows for faster drying.
5. Incubation at 56°C heat-fixes the section to the slide. The section will fall off the slide during staining if not properly fixed to the slide.
6. Because the slide has no cover-slip, histologic features may not be easily identified. Cellular details can be better visualized by pipetting 100% ethanol onto the tissue section.
7. Large microdissected areas can be lifted directly from the slide with a clean pair of forceps and placed into 100 μ l of buffer. We generally perform LM at 4 \times magnification (after careful inspection at 10 \times) when working with frozen breast tissue. If the laser tends to drift from the user-defined cut lines, calibrate the laser periodically. With Laser Microdissection LMD software version 4.4, the laser control settings we typically use at 4 \times are aperture, 15; intensity, 44; speed, 6 for clean cuts; offset, 18; bridge, medium; aperture differential, 6.
8. The lysis solution and wash solutions may be stored at 4°C or room temperature, but be sure to warm the reagents to room temperature before beginning the protocol.
9. The Pico Ladder should only be heated when first diluted and aliquoted and may not run properly if heated multiple times.
10. Completely submerge the foil containing the microdissected tissue in the lysis solution to ensure complete cell lysis and inactivation of endogenous RNases. If this is not achieved by centrifugation, push the foil into the buffer using a pipette tip.
11. To isolate both large and small RNA species including tRNAs or microRNAs, add 129 μ l of 100% ethanol rather than 52 μ l.
12. When transferring the sample to the filter, be sure not to transfer large pieces of foil that may block the flow of liquid through the filter.

13. To increase accuracy when pipetting hot liquids, prewet the pipette tip before pipetting the 95°C water and applying it to the filter.
14. To easily remove the DNase Inactivation Reagent from the RNA and maximize RNA recovery, centrifuge the sample to pellet the Reagent. Transfer ~20 µl of RNA to a new tube, centrifuge the tube containing the Reagent for an additional 30 s to 1 min, then transfer the remaining RNA using a small bore pipette tip (10XL tips work well) without disturbing the DNase Inactivation Reagent pellet.
15. When running a Pico or Nano Chip, ensure that the reagents have not expired and that no more than 2 months have passed since the gel was filtered. Expired reagents can adversely affect the run and can cause aberrations to the baseline of the electropherogram.
16. When cleaning the Bioanalyzer electrodes prior to running a Pico or Nano Chip, fill the electrode cleaner with nuclease-free water, not RNaseZap®. The Pico Chip is very sensitive and even minute amounts of RNaseZap® can interfere with proper function.
17. When loading the Pico or Nano Chips, do not use the blow-out function of the pipette as this may introduce air bubbles that may interfere with the chip running properly.
18. Most RNA samples isolated from frozen tissue via laser microdissection have a RIN (RNA Integrity Number) greater than 7, which is acceptable for downstream applications. Samples with a lower RIN (between 6 and 7) may be usable. Repeat the RNA isolation if significant degradation has occurred. Remove the sample from the study if subsequent isolations do not show improved RNA quality.
19. The pin set on the Bioanalyzer will need periodic maintenance – remove and clean thoroughly with RNaseZap®, then rinse thoroughly with nuclease-free water.
20. The final volume of this dilution may be adjusted based on the number of samples that will be amplified at one time. For example, if amplifying ten or more samples, prepare 40 µl of the fifth dilution by mixing 4 µl of the fourth dilution with 36 µl of nuclease-free water.
21. We typically use 10 ng of RNA as input for the first round of amplification; however, we have amplified as little as 2 ng with more than sufficient yields of second round aRNA.
22. If the starting amount for amplification is greater or less than 10 ng, adjust the volume of the Poly-A Controls proportionately. For example, if starting with 5 ng of RNA, add 1 µl of the fifth dilution to the sample.

23. Ensure that the thermal cycler lid is also cooled to 16°C. Exposure of the reaction to higher temperatures will lead to inefficient second-strand cDNA synthesis and will compromise aRNA yield. If the lid temperature cannot be adjusted to 16°C, turn the lid temperature off or incubate with the lid off.
24. We have started the second round of amplification with as little as 350 ng of first round aRNA with successful results. Any remaining aRNA from the first round of amplification can be stored at -80°C for future use.
25. Because the samples have just been heated to 94°C, it is not necessary to heat denature them at 70°C. The heat-denatured ladder can be used in this run without reheating. The same gel-dye mix that was prepared to run the Nano Chip for the second round aRNA can be used to run the Nano Chip for the fragmented aRNA if both chips are run on the same day.
26. Before placing the arrays in the hybridization oven overnight, cover each septum with a small piece of tape to prevent the sample from leaking out of the array.
27. Arrange the arrays in the hybridization oven with a balanced configuration to prevent undue stress to the motor.
28. We typically reverse transcribe sufficient aRNA to run all of the TaqMan® Gene Expression Assays that we have defined for the study so that additional reverse transcription reactions on the same sample are not necessary.
29. We recommend using TaqMan® Gene Expression Assays with the suffix “_ml” if possible, as these assays amplify regions spanning exon junctions and will not amplify genomic DNA that may contaminate the RNA sample. For “_gl” or “_sl” assays, include a control with no reverse transcriptase when performing the reverse transcription reaction and subsequent PCR to ensure that amplification is due to the presence of RNA transcripts and not genomic DNA contamination.
30. 10 ng of cDNA usually produces good results; however, when working with certain low abundance transcripts, the starting amount of cDNA may need to be increased and the volume of water in the master mix adjusted accordingly.
31. In some cases, the chosen reference RNA may not express a particular transcript of interest and an alternative reference RNA will need to be selected.
32. To calculate relative transcript levels using the Comparative C_t Method:
 - (a) Determine the ΔC_t as follows:

$$\Delta C_t = C_{t\text{target}} - C_{t\text{reference}}$$

where the target is the gene of interest and the reference represents an endogenous control such as actin or GAPDH.

(b) Calculate the $\Delta\Delta C_t$ using the following formula:

$$\Delta\Delta C_t = \Delta C_{t \text{ test sample}} - \Delta C_{t \text{ calibrator}}$$

(c) The fold change relative to the calibrator is $2^{-\Delta\Delta C_t}$.

As an alternative to the Comparative C_t Method, relative levels of gene expression can be determined using the Relative Standard Curve Method. The following document describes both the Comparative C_t Method and the Relative Standard Curve Method:

Applied Biosystems. Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, available at: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf.

33. This protocol has been modified by the manufacturer. The current RecoverAll™ protocol reduces the volume of digestion buffer from 400 to 100 μ l, shortens the incubation from 3 h at 50°C to 15 min at 50°C followed by 15 min at 80°C, reduces the volume of isolation additive from 480 to 120 μ l and the volume of 100% ethanol from 1.1 ml to 275 μ l, and lowers the temperature of the eluant from 95°C to room temperature (22–25°C).

Acknowledgments

This work was supported by the United States Department of Defense (Military Molecular Medicine Initiative MDA W81XWH-05-2-0075, protocol #01-20006) and was performed under the auspices of the Clinical Breast Care Project, a joint effort of many investigators and staff members. The opinion and assertions contained herein are the private views of the authors and are not to be construed as official or as representing the views of the Department of the Army or the Department of Defense.

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Laser Capture Microdissection

Methods and Protocols

Murray, G.I. (Ed.)

2011, XVII, 493 p. 97 illus., 1 illus. in color., Hardcover

ISBN: 978-1-61779-162-8

A product of Humana Press