RNA from Air-Dried Frozen Sections for RT-PCR and Differential Display


Evaluation of in vivo gene expression in specific types of cells is difficult because animal tissues are complex mixtures of many cell types, and RNA extracted from homogenized fresh whole tissue is derived from all cell types present. In an attempt to improve specificity, RNA has been isolated from specific cells microdissected from fixed, paraffin-embedded tissue sections (2,6), but the yield and quality of RNA have generally been suboptimal. Here we report an alternative method for recovering high-quality RNA from specific cells microdissected from air-dried frozen histological sections of unfixed tissue and its analysis by reverse-transcription polymerase chain reaction (RT-PCR) and by the relatively new technique of differential display RT-PCR (DDRT-PCR) (1,3,4).

To prepare air-dried frozen sections, unfixed tissue is quickly snap-frozen or removed from the freezer and adhered to a cryostat chuck. It is critical to avoid thawing in order to inhibit RNase activity. Sections are cut at 20 µm and thaw-mounted on glass slides. The slides are dried in a 37°C incubator for 5 min and either microdissected immediately or stored airtight with a desiccant at < -70°C. Typical equipment for microdissection includes an inverted microscope (with 4X and 10X objectives) and an attached mechanical micromanipulator for holding and manipulating the cutting tool (e.g., a 30-gauge hypodermic needle superglued into a glass microcapillary tube). Specific cells can be visualized without staining the tissue if the microscope contrast is high (e.g., by unfocusing the condenser or using phase-contrast rings) and if an adjacent hematoxylin- and eosin-stained section is used as a guiding template. Alternatively, staining briefly (30 s) with aqueous hematoxylin and quickly re-drying the slide allow for better direct visualization and only slightly decrease the yield of RNA. Accurately separating different types of cells requires familiarity with the histopathological features of the tissue. Approximately 1 x 10⁶ cells are needed to obtain sufficient RNA to run one primer pair in DDRT-PCR or RT-PCR. Thus, dissecting one to several slides may be required depending on the target cell distribution and density. Obtaining sufficient cells can be tedious and time-consuming if the target cells are rare and/or scattered in small groups. Harvesting enough cells can be greatly facilitated by pre-selecting specimens containing relatively large areas of apposed target cells. It is possible to routinely prepare samples enriched to >95% target cellularity, although 100% purity is nearly impossible due to intermingled capillary endothelium, fibroblasts, lymphocytes, etc.

Once the tissue is harvested, RNA can be extracted from air-dried frozen histological sections of breast tissue using methods previously described (6). Following isopropanol-precipitation, sample RNA pellets are washed with 200 µL 80% ethanol, dried under a vacuum for 5 min, or alternatively, the pellets are swabbed with a cotton-tipped applicator, quickly air-dried and then resuspended in 10 µL of 10X RT buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 30 mM MgCl₂).

RT of sample RNA was carried out in 0.5-µL thin-wall microcentrifuge tubes (PGC Scientific, Gaithersburg, MD, USA) in a total volume of 96 µL RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 3 mM MgCl₂) along with 1 mM dNTPs, 0.5 nmol of random hexamer primers and 5 µL of sample RNA (the amount of RNA utilized in the reaction should be empirically determined for different tissues sources). Samples were denatured at 94°C for 2 min, cooled to 42°C for 2 min and then incubated at 42°C for 45 min following the addition of 2.0 U of avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, St. Petersburg, FL, USA). Following denaturation of the reverse transcriptase at 94°C for 3 min, 0.1 nmol each of sense and antisense primers designed to amplify a small region of the human estrogen receptor (ER) and 2.5 U of AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) were added. The samples were covered with 100 µL of mineral oil, and the reaction tubes were heated at 94°C for 1 min. PCR was then carried out in a Perkin-Elmer cycler using 40 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. These parameters have proven to be optimum when amplifying this fragment of the ER gene. However, cycling parameters should be optimized for each gene-specific primer pair used. These cycling steps were then followed by a single extension cycle of 94°C for 1 min, 55°C for 2 min and 72°C for 7 min. The Taq polymerase was then denatured at 98°C for 10 min, and the samples were cooled to 27°C. Ten microliters of each sample were loaded onto a 5% mini-polyacrylamide gel (Bio-Rad, Hercules, CA, USA), separated by electrophoresis and then stained with a 1% solution of ethidium bromide.

As shown in Figure 1, PCR products of the expected size of 170 bp were amplified from four breast tissue samples using oligonucleotide primers specific for separate exons of the ER. It is advisable to choose primers across

![Figure 1. RT-PCR of RNA recovered from air-dried frozen sections.](Image)
intrinsic regions for the gene-specific amplification so that any contaminating genomic DNA present in the RNA preparation will not be amplified. RNA isolated directly from the MCF-7 human breast cancer cell line (5 µg) was amplified as a positive control and yielded identical bands to those seen in Figure 1 (data not shown). Amplifications performed with water in the place of sample, and also reactions lacking reverse transcriptase were carried out as negative controls and gave no amplification products. All PCRs were carried out with equipment and reagents allocated for strict usage in PCR experiments only.

The methodology used for differential display was based on that reported originally by Liang and Pardee (4). RT of each sample was carried out in a total of 50 µL in RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 3 mM MgCl₂), 50 µM dNTPs, 1 pmol of an oligo(dT) 11-mer antisense oligonucleotide primer (T11CA) and either 1.0 µL of sample RNA (again, the amount of RNA added to the reaction should be empirically determined with each tissue source of RNA) or 2 µg of control RNA from MCF-7 cells. Samples were heated to 65°C for 5 min, followed by a 3-min temperature equilibration at 40°C. Five units of AMV reverse transcriptase were added, and the samples were incubated at 40°C for 57 min, followed by denaturation at 94°C for 3 min. The samples were then placed on ice until amplified. We have also found that RT reactions such as these can be maintained at -70°C for up to 6 weeks and they can still be utilized for PCR

Figure 2. DDRT-PCR results from air-dried frozen and permanent (perm.) sections of the endocervix. Reverse-transcribed cDNA was amplified by PCR from histological sections of endocervix using a T11CA antisense primer and a random 10-mer sense primer as described in the text. In Panel a, the large number of bands in the air-dried frozen samples (lanes 3 and 4) demonstrates that DDRT-PCR can be used for analysis of RNA species as large as 600 bp extracted from tissues of this type. Tissue from the sample in lane 3 was stained with hematoxylin. MCF-7 cell RNA (lane 1) and RNA from a frozen section of endocervix (lane 2) were used as positive controls. In Panel b, DDRT-PCR was performed on RNA recovered from formalin-fixed, permanent (paraffin-embedded) sections (6) of the endocervix with (+) or without (-) hematoxylin staining. Negative controls lacking RT generated no significant banding patterns (not shown). M = MspI-digested pBR322 plasmid (Promega).
amplification for the purposes of differential display (data not shown). PCR amplification of cDNAs was again done in thin-walled microcentrifuge tubes that contained 1.8 µL of PCR amplification buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 10 mM MgCl2), an additional 12 µM dNTPs, 4.3 pmol of additional T11CA primer, 5 pmol of a random 10-mer sense oligonucleotide primer and 16 µL PCR-quality water. To this mixture, 7 µCi of [35S]dATP (NEN Life Science Products, Boston, MA, USA), 2 µL of the RT reaction and 2.5 U of Taq DNA polymerase were added, followed by a layer of 50 µL of mineral oil. Sample tubes were heated at 94°C for 1.5 min, and amplification was performed for 40 cycles at 94°C for 30 s, 42°C for 1.5 min and 72°C for 30 s, followed by a final extension at 72°C for 5 min, inactivation at 98°C for 10 min and a final cooling to 27°C. Following addition of 5 µL of formamide loading buffer, each sample was heated at 80°C for 3 min, 10 µL loaded onto a 5% Long Ranger™ sequencing gel (AT Biochem, Malvern, PA, USA) and electrophoresed at 70 W for 3 h. Gels were dried under a vacuum and a Postdoctoral Fellowship from the Department of Defense to C.G.C. Address correspondence to Suzanne A.W. Fuqua, Department of Medicine/Oncology, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284, USA.

As shown in Figure 2a, DDRT-PCR using RNA from MCF-7 control RNA (lane 1), a frozen tissue section of the endocervix (lane 2) and sections of the endocervix that were air-dried at 37°C with or without hematoxylin staining (lanes 3 and 4, respectively) were separated on a 5% acrylamide sequencing gel and exposed to X-ray film. The uniform appearance of bands across all four samples suggests that none of the RNA samples in this experiment have undergone significant degradation and that RNA of sufficient quality for this type of analysis can be recovered from air-dried frozen samples. This is of particular interest, since we had initially expected RNases to greatly impact our ability to recover useful amounts of RNA from air-dried tissue sections, especially those stored at 37°C. In addition, formalin-fixed, paraffin-embedded permanent sections of the endocervix (Figure 2b) also yielded RNA by this published RNA isolation procedure (6), which was readily amplified by DDRT-PCR. However, we have found that fixation should be performed as quickly as possible to avoid RNase digestion. Also, as seen in Figure 2, a and b, we have found that hematoxylin staining does not appear to significantly alter the quality of RNA extracted from archival tissue sections and allows for much easier microdissection. It is very important that the RNA is as free as possible of contaminating DNA. An RNase treatment can be added to the RNA extraction procedure (6) using the suggested manufacturer’s procedure (RNase Q™, Promega, Madison, WI, USA) before DDRT-PCR. We also include a reaction lacking RT to ensure that the banding pattern seen is cDNA amplification (data not shown).

Evaluating gene expression by differential display may be relatively simple if the source of RNA is specific and abundant, such as with cell lines. DDRT-PCR is an attractive alternative to subtraction hybridization cloning of RNA amplified from microdissected frozen materials as described by Luqmani and Lymboura (5). In this instance (5), RNA was obtained from breast cancer tissues kept below -20°C during the microdissection. This is in contrast to that reported here, where RNA is obtained from air-dried frozen histological sections, making the actual microdissection process much easier. Animal tissues such as human tumors may contain sufficient RNA for amplification, but it is derived from many cell types, which are present in varying proportions. Specific cells can be obtained from heterogeneous animal tissues by microdissecting them from histological sections viewed under a light microscope. Histological sections are typically prepared from formalin-fixed, paraffin-embedded tissue and stained with hematoxylin and eosin. Unfortunately, the RNA extracted from this type of tissue is sometimes too fragmented and meager for routine differential display. However, we show that adequate amounts of high-quality RNA can be obtained from air-dried frozen histological sections. The relative absence of water in these sections is apparently sufficient to inhibit endogenous RNase activity, making it possible to amplify routinely RNA sequences larger than 600 bp.

REFERENCES


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