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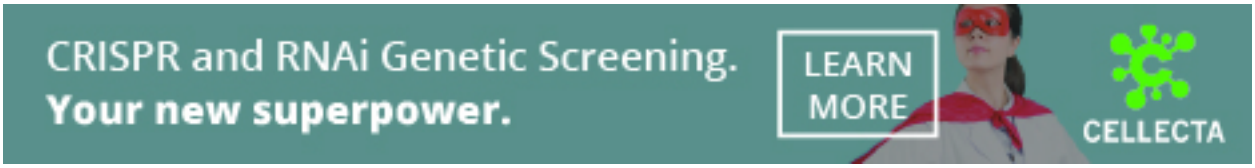

Modified Tissue Pulverization Technique and Evaluation of Dihydrofolate Reductase Amplification as a Pan-tissue RT PCR Control

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When processing large numbers of tissue samples for amplification by reverse transcriptase polymerase chain reaction (RT PCR), certain factors must be considered. Reuse of tissue homogenization equipment and handling of multiple samples increase the probability of contamination, leading to false-positive reactions. Additionally, studies that examine a variety of tissues or cell types require a universally expressed gene as an amplification control. These concerns were initially addressed as a result of our attempts to amplify extremely low levels of persistent coxsackievirus RNA from skeletal muscle. Isolation of intact RNA from skeletal muscle is difficult because of the small amount of tissue available and the comparatively low levels of RNA. These drawbacks are compounded by the presence of connective tissue, which makes tissue homogenization difficult. To overcome these difficulties, we developed a modification of a previously reported method[1] that requires little equipment, minimizes the potential for contamination, and facilitates the isolation of high-quality RNA from frozen mouse muscle. It has also proved effective for pulverization of a wide variety of tissues. We then used this RNA to test the feasibility of using dihydrofolate reductase (DHFR) as a pan-tissue amplification control.

Mouse tissue was harvested, placed in cryotubes, flashfrozen in liquid nitrogen, and stored at −70°C. During pulverization, all tissue samples were held on dry ice. To prevent cross-contamination, a separate nylon forceps was used to handle each tissue sample. Before reuse, forceps were washed, soaked in 0.5 N NaOH to hydrolyze contaminating RNA, rinsed in water, and autoclaved. For each sample, an envelope of aluminum foil was prepared, which was four layers thick and at least five times larger than the tissue sample. Just before use, the envelope was dipped in liquid nitrogen and the tissue was placed inside. This was dipped in liquid nitrogen again and placed on a foil-covered 2.5-cm-thick iron plate stored at −70°C and positioned on a bed of dry ice. The tissue was completely pulverized by striking it several times with a hammer. The pulverized tissue formed a disk that could be picked up with forceps after flexing the foil and transferred to a 1.5-ml microcentrifuge tube containing 520 μl of solution D.[2] The tissue was immediately vortexed on medium-high speed for 10–20 sec, and then microcentrifuged for 10 sec to pellet insoluble debris. A 500 μl volume was transferred to a fresh tube, leaving behind tissue debris that might trap RNases.[3] At this point the sample was kept at room temperature (for a maximum of 30 min) until remaining samples were processed. RNA isolation was continued as described.[2]

RT PCR amplification of DHFR employed a 25-mer sense primer DHFR-S2 (5′-TCG ACC ATT GAA CTG CAT CGT CGC C-3′) from exon I and a 26-mer antisense primer DHFR-A1 (5′-GGA ATG GAG AAC CAG GTT TTC CTA CC-3′) from exon III, based on the published sequence for mouse DHFR.[4] These primers span nucleotides 6–185 of the mRNA. Reverse transcription of DHFR was performed in a 20-μl reaction containing 10 pmoles of DHFR-A1, 20 units of RNasin, and 50 units of Moloney murine leukemia virus (MMLV) RT (GIBCO-BRL, Bethesda, MD), with buffer components as recommended by the manufacturer. The reaction was incubated at 37°C for 1 hr, 95°C for 5 min, and cooled to 5°C. PCR was performed by adding 10 pmoles of DHFR-S2 and additional reagents to obtain final reaction conditions of 1.5 mM $MgCl_2$, 40 mM KCl, 10 mM Tris (pH 8.3), and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in a final volume of 100 μl. Thirty-five cycles of 94°C for 1 min, 50°C for 1.5 min, and 72°C for 1.5 min were followed by a final extension at 72°C for 8 min.

On the average, we recovered 26 μg of RNA from 30 mg wet weight of mouse hindquarter skeletal muscle, with an $OD_{260/280}$ ratio of 1.8. This is comparable to reported muscle RNA yields.[1] RNA was obtained from samples of mouse muscle weighing up to 100 mg without increased degradation or loss. Densitometric tracings of rRNA bands yielded an average 28S/18S ratio of 1.8[5] The quality of RNA extracted from mouse spleen, liver, kidney, diaphragm, thymus, heart, brain, spinal cord, and small intestine was comparable to that of mouse skeletal muscle. One exception was the pancreas, which had a 28s/18s ratio of 1.1 and is known to possess high levels of RNase. Disruption of muscle by other methods such as vortexing with glass beads or hand-held homogenizers yielded similar recoveries of RNA but of less consistent quality. Aerosolization was also more

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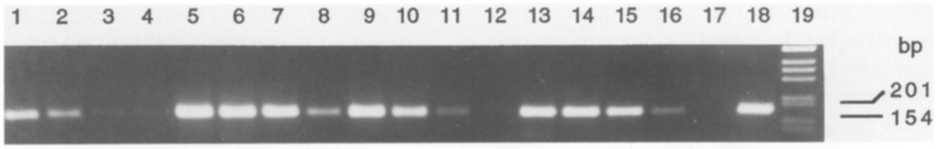

FIGURE 1 Titration of RNA in DHFR amplification reaction. DHFR product (180 bp) from RT PCR containing 200, 20, 2, and 0.2 ng of RNA from skeletal muscle (lanes *1–4*), spleen (lanes *5–8*), brain (lanes *9–12*), liver (lanes *13–16*), template-free negative control (lane *17*), positive control standard (lane *18*), and 1-kb DNA ladder (BRL) (lane *19*).

likely because of foaming in the glass bead method and displacement of liquid during microtube homogenization, both of which may exacerbate cross-contamination.

Using 2 μg of total RNA as template, DHFR was amplified successfully from all sources of tissue described above, including the pancreas. A 180-bp band was observed after running 20 μl of RT PCR product on a 4% NuSieve 3 : 1 agarose gel (FMC, Rockland, ME). Titration of the template RNA for several different tissues showed that a clear positive reaction could be obtained with as little as 20 ng of skeletal muscle RNA, 2 ng of brain RNA, and 0.2 ng of spleen or liver RNA (Fig. 1). Levels of DHFR transcripts are particularly low in differentiated muscle and have been estimated at 0.7 copies per cell while actively dividing cells express higher levels.[6] Amplification of DHFR was equally effective using random hexamers in place of DHFR-A1 to prime the RT reaction (results not shown). This allows both test and control amplification reactions to be run on the same cDNA sample.

β-Actin mRNA is commonly used as an amplification control, although the large number of processed β-actin pseudogenes can give rise to false-positive reactions.[7] Several DHFR pseudogenes have also been described but were not amplified here because no product was formed when RT was omitted from reactions containing 2 μg of skeletal muscle, brain, spleen, or liver RNA. A representative sample is shown in Figure 2. A *Hae*III digest of the DHFR product from all tissues tested yielded the expected 110- and 70-bp bands.

Thus, DHFR is a suitable amplification control for use with a wide variety of tissues. It is especially useful as a control for rare transcripts in muscle. Our DHFR primers were selected to yield a product similar in size to the coxsackievirus RT PCR product. Because other primer sequences have been published for mammalian DHFR transcripts, the amplification control can be easily tailored to produce products of the desired size. Subsequently, these methods worked equally well when applied to frozen human muscle biopsy specimens using primers from the same regions but with homology to human DHFR. Thus, this approach should also be useful for retrospective analyses of frozen clinical specimens.

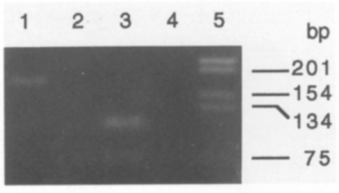

FIGURE 2 Testing for DHFR pseudogene amplification and diagnostic *Hae*III digest of DHFR RT PCR product. (Lane *1*) RT PCR product from 0.2ng of liver RNA as reference; (lane *2*) 2 μg of spleen RNA subjected to RT PCR without addition of RT; (lane *3*) *Hae*III digest of DHFR product; (lane *4*) template-free negative control; (lane *5*) 1-kb DNA ladder (BRL).

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