

Evaluation of Methods for RNA Isolation and cDNA Synthesis Using Small Articular Cartilage Samples.

Summary: Aberrations in the normal balance of chondrocyte metabolism are thought to play an integral role in degeneration of articular cartilage and subsequent arthritis. It is believed that, through a greater understanding of the normal and pathologic control mechanisms used by the chondrocytes to maintain the articular cartilage, a better understanding of the disease process and potential treatments would be obtained. The objective of this study was to compare the different methods of cartilage RNA extraction and cDNA synthesis. From this work, an optimal method that will reliably yield DNA/RNA even from small samples obtained at surgery has been determined. Furthermore, usable DNA/RNA has been obtained from specimens that have been collected and stored in paraffin, allowing flexibility to use historic samples with state-of-the-art technology.

Osteoarthritis (OA) is a major problem for humans, as well as domestic animals such as the dog and horse. By definition, arthritis is pathology of hyaline cartilage, a highly specialized connective tissue composed of a small number of chondrocytes surrounded by a large extracellular matrix.

Aberrations in the normal balance of chondrocyte metabolism are thought to play an integral role in degeneration of articular cartilage and subsequent arthritis. It is believed that, through a greater understanding of the normal and pathologic control mechanisms used by the chondrocytes to maintain the articular cartilage, a better understanding of the disease process and potential treatments would be obtained. The present understanding of these processes has been somewhat hampered at the molecular level due to relatively low chondrocyte numbers, (cartilage contains only 1-2% chondrocytes), the high concentration of proteoglycans that many times co-purify with RNA, coupled with the fact that cartilage biopsies are usually only 5-10 mg. These factors can make the study of gene expression patterns very difficult.

One of the most common methods of extracting RNA is to homogenize the tissue in Trizol®. This can be difficult when working with tissue such as cartilage that does not homogenize easily. Longer periods of homogenization can cause an increase in temperature, which can lead to degradation of the RNA. Another method for extracting RNA is snap-freezing tissue and using the Qiagen RNeasy Kit. Again, this can be difficult with tissue like cartilage that is hard to crush with a mortar and pestle. The advantage to this method is that it is a relatively short procedure allowing for greater sample throughput than other methods. Finally, RNA can be extracted from paraffin embedded tissue using a Qiagen DNeasy Tissue Kit. The disadvantage of this method is that cartilage has to remain at 56°C for a

longer period of time as it is difficult to lyse. Again, the longer the RNA spends at a higher temperature, the greater the chance of degradation occurring. Therefore, the objective of this study was to compare the different cartilage RNA extraction and cDNA synthesis methods. The research work was performed by Andi Hume, under the supervision of Dr. David Frisbie.

Samples were taken from articular cartilage with different chondrocyte densities, the medial femoral condyle, both trochlear ridges of the distal femur and the distal third metacarpal condyle of three horses. The samples, which were approximately 5 mm square, were either embedded in paraffin (n=47), snap-frozen (n=10) or stored in 1 ml of Trizol® (n=54). Paraffin sections were extracted using the Qiagen DNeasy Tissue Kit, snap-frozen samples were extracted using the Qiagen RNeasy Mini Kit, and Trizol® samples were homogenized and extracted according to the manufacturer's specifications. The price per sample was calculated for each RNA extraction method. cDNA was created using either 100U Superscript II RT (Method 1) or 200U MMLV-RT (Method 2). cDNA was diluted to a final volume of 20 µl for both methods. To assess the effect of further dilution when a greater volume of cDNA is needed, some cDNA aliquots synthesized using Method 1 were diluted to a 100µl final volume. The RNA synthesized into cDNA using Method 1 was treated with 150U RNase-free DNase to test/ensure no genomic contamination of the mRNA had occurred. The ABI PRISM 7700 Sequence Detection System was used to detect the housekeeping gene GAPDH. GAPDH expression was evaluated in all samples by comparing their linearized cycle threshold (C_T) values ($2^{(40-C_T)}$). C_T, or cycle threshold value is the PCR cycle number at which amplification of the target sequence occurs. The lower the C_T value, the

Summaries: Focus 2

Early Diagnosis of Bone and Joint Disease

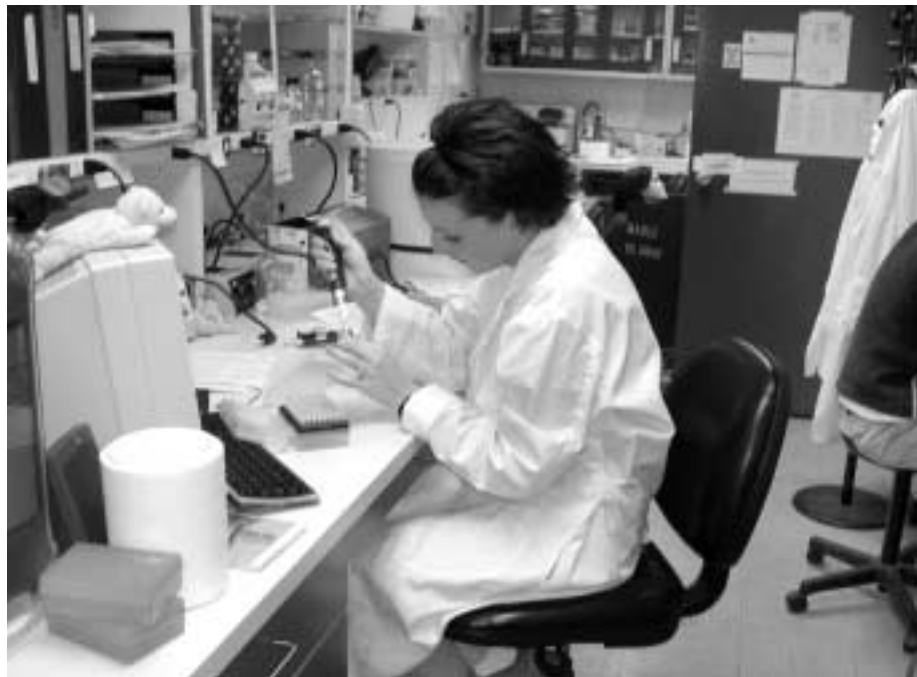
greater the level of gene expression. A Mixed model analysis of variance was used to evaluate the fixed effects of tissue harvest location, fixation/storage protocol and cDNA synthesis method. When appropriate, a Least Squares means procedure was used to make individual comparisons and a p-value <0.05 was considered significant.

The methods of RNA extraction and cDNA synthesis were significantly different ($P=0.0001$), and, although the location from which the cartilage was collected showed a trend for differences, they were not significantly different. There was a significant media effect, as Trizol[®] had lower average C_T values than both snap-frozen and paraffin extracted samples. The average C_T for cDNA synthesis Method 2 was 25.85 compared to 30.86 for Method 1 analyzing the 20 μ l aliquots. No significant difference was noted in the average C_T values when samples using cDNA synthesis Method 1 were analyzed using either 20 μ l or 100 μ l volume (30.86 versus 30.87, respectively).

The cost of the Trizol[®] extraction procedure was \$1.00-\$2.00 cheaper per sample than using either of the Qiagen extraction procedures.

Although all of the methods evaluated here have their unique benefits and are viable methods, we have determined that, based on time, cost and reliability, using Trizol[®] to extract RNA from articular cartilage is the optimal method when paired with cDNA synthesis Method 1. From this work, we have determined an optimal method which will reliably yield DNA/RNA even from small samples obtained at surgery. Furthermore, we have determined that we can obtain usable DNA/RNA from specimens that have been collected and stored in paraffin, allowing us flexibility to use historic samples with state-of-the-art technology.

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Andi Hume works in the lab.