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Comparison of extraction techniques for amniotic fluid supernatant demonstrates improved yield of cell-free fetal RNA

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Fetal cell-free mRNA in amniotic fluid (AF) is a valuable source of information regarding the developing fetus. Nucleic acids isolated from AF provide novel information compared to other sources of cell-free nucleic acids, such as maternal plasma (Zhong *et al.*, 2006). Genomic and transcriptomic information from AF is of special interest because it derives solely from the fetus, and may therefore offer unique perspectives. The discovery-driven approach of using transcriptomic analysis has contributed novel information about the pathogenesis of several genetic disorders, such as Down syndrome and polyhydramnios in the setting of twin–twin transfusion syndrome (Larrabee *et al.*, 2005; Slonim *et al.*, 2009).

The successful extraction of fetal RNA from cell-free AF supernatant and its subsequent hybridization to oligonucleotide microarrays has already demonstrated the wealth of new information this nucleic acid source has to offer (Hui and Bianchi, 2010). A possible impediment to the expanded utilization of AF is the cost and labor associated with extraction of DNA and RNA. A method using the combination of Trizol and the RNeasy Maxi Kit (QIAGEN, Hilden, Germany) has been used in our laboratory to successfully extract RNA from AF supernatant. However, this method does not allow the simultaneous processing of large numbers of samples and includes several possible sources of error, such as the division of samples into smaller aliquots and a precipitation step. Recently, a new kit was made commercially available to extract nucleic acids from human plasma or serum, the QIAamp Circulating Nucleic Acid Kit (QIAGEN). This kit utilizes a vacuum extraction method that

allows throughput of large volumes on one mini column and eliminates the need for precipitation of the final sample. The purpose of this study was to compare RNA extraction from AF using this new method to our laboratory's previously reported standard methods (Larrabee *et al.*, 2005; Slonim *et al.*, 2009).

This study was approved by the Tufts Medical Center Institutional Review Board. Five residual samples of AF supernatant that were obtained for routine clinical indications at Tufts Medical Center were used [46,XY ($n = 2$) and 46,XX ($n = 3$)]. The gestational ages ranged from 15 4/7 weeks to 17 3/7 weeks. After the initial collection of AF, samples were processed and frozen at -80°C within 12 h. The samples had been stored at -80°C for up to 9 months before RNA extraction was performed. The samples of 10 mL were each split into two 5 mL aliquots for the two different extraction methods.

For the standard approach, total RNA was extracted from one 5 mL aliquot of each AF sample using 15 mL of Trizol LS Reagent (Invitrogen) and 4 mL of chloroform per sample. Subsequent reactions were divided into smaller fractions, consisting of 750 μL of Trizol, 250 μL of AF supernatant sample, and 200 μL of chloroform per reaction in order to utilize the microcentrifuge necessary for the protocol. This method resulted in approximately 20 different reactions per 5 mL sample. Extracted RNA was then purified using the RNeasy Maxi Kit (QIA-GEN) as per the manufacturer's protocol. RNA was precipitated from the original volume of approximately 650 μL and then rehydrated in 20 μL of RNase-free water. Briefly, 3 M NaOAc and 100% ethanol were added to the sample, the sample incubated at -20°C for 4 h, the RNA was pelleted by centrifugation and the precipitated RNA was washed by adding 80% ethanol, followed by centrifugation and rehydration of the RNA.

For the new protocol, total RNA was extracted from the second 5 mL aliquot from all samples using the QIAamp Circulating Nucleic Acid Kit (QIAGEN), according to the 'Isolation of Circulating Nucleic Acids from Plasma or Serum' protocol as recommended by the manufacturer. Briefly, the sample was lysed and then applied to a column via a vacuum pump. The RNA (bound to the spin column) was then washed three times and eluted with 20 μL of RNase-free water. On-column DNase digestion (RNase-Free DNase Set, QIAGEN) was performed during extraction in both methods.

All extracted RNA samples were then stored at -80°C for approximately one week prior to real-time quantitative reverse transcriptase (qRT) PCR amplification. Real time qRT-PCR was performed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with primer and probe sequences as previously reported (Maron *et al.*, 2008), using an ABI 7900 Sequence Detection System with TaqMan One-Step RT-PCR Master Mix reagents kit (Applied Biosystems, Foster City, CA, USA). All qRT-PCR experiments were performed in duplicate with negative controls on each plate. The quality of total RNA in extracted RNA samples was assessed by an Agilent Bioanalyzer 2100 (Foster City, CA, USA).

All statistical analyses were performed with SPSS 11.5 software (SPSS Inc., Somers, NY, USA). Independent *t*-tests were performed between the mean cycle of amplification of *GAPDH* between the samples extracted using the two different methods to determine if there

were statistically significant expression differences. Statistical significance was set at a threshold level of $p < 0.05$.

Our results demonstrated that RNA extracted using the new method amplified at a significantly earlier cycle threshold than RNA extracted using the standard method ($p = 0.001$, Figure 1). This demonstrates significantly improved quantity of extracted RNA using the newer method. In order to gather qualitative data, the bioanalyzer results for both extraction methods were analyzed. These results were inconclusive, with similar quality RNA obtained from both methods. We speculate this is due to the overall low concentration and degraded nature of the RNA obtained from both methods. These findings demonstrate that neither method has a significant effect on the quality of isolated RNA. This impeded qualitative comparisons but still allowed qRT-PCR amplification. The new extraction method also lowered the risk of pipetting error by eliminating the step of splitting the samples into 1.5 mL tubes and a precipitation step. The protocol changes also significantly decreased the time to process the samples, with the new method requiring only 2.5 h, whereas the standard method requires 8.5 h. The new method is also more cost effective, with the total cost of the standard method being \$53.33/sample compared to \$21.11/sample for the new method (\$USD).

In summary, the new method of isolating cell-free RNA from AF samples using the QIAamp Circulating Nucleic Acid Kit represents overall improvement in many ways. It is both more cost effective and less labor intensive than the standard method of RNA extraction from AF. By increasing the quantity of extracted RNA this new method makes downstream applications more accessible and robust, thereby offering an improved workflow to further understand both normal fetal development and the pathogenesis of many genetic disorders.

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Figure 1. Comparison of the mean Ct values of each extraction method. RNA extracted using the QIAamp Circulating Nucleic Acid Kit (CNA Kit) amplified significantly earlier than RNA extracted using Trizol and the Maxi Kit ($p = 0.001$). Bars represent standard deviation