Purification of cardiac myocytes from human heart biopsies for gene expression analysis

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Kosloski LM, Bales IK, Allen KB, Walker BL, Borkon AM, Stuart RS, Pak AF, Wacker MJ. Purification of cardiac myocytes from human heart biopsies for gene expression analysis. Am J Physiol Heart Circ Physiol 297: H1163–H1169, 2009. First published July 17, 2009; doi:10.1152/ajpheart.00118.2009.—The collection of gene expression data from human heart biopsies is important for understanding the cellular mechanisms of arrhythmias and diseases such as cardiac hypertrophy and heart failure. Many clinical and basic research laboratories conduct gene expression analysis using RNA from whole cardiac biopsies. This allows for the analysis of global changes in gene expression in areas of the heart, while eliminating the need for more complex and technically difficult single-cell isolation procedures (such as flow cytometry, laser capture microdissection, etc.) that require expensive equipment and specialized training. The abundance of fibroblasts and other cell types in whole biopsies, however, can complicate gene expression analysis and the interpretation of results. Therefore, we have designed a technique to quickly and easily purify cardiac myocytes from whole cardiac biopsies for RNA extraction. Human heart tissue samples were collected, and our purification method was compared with the standard nonpurification method. Cell imaging using acridine orange staining of the purified sample demonstrated that >98% of total RNA was contained within identifiable cardiac myocytes. Real-time RT-PCR was performed comparing nonpurified and purified samples for the expression of troponin T (myocyte marker), vimentin (fibroblast marker), and α-smooth muscle actin (smooth muscle marker). Troponin T expression was significantly increased, and vimentin and α-smooth muscle actin were significantly decreased in the purified sample (n = 8; P < 0.05). Extracted RNA was analyzed during each step of the purification, and no significant degradation occurred. These results demonstrate that this isolation method yields a more purified cardiac myocyte RNA sample suitable for downstream applications, such as real-time RT-PCR, and allows for more accurate gene expression changes in cardiac myocytes from heart biopsies.

real-time reverse transcriptase-polymerase chain reaction; vimentin; troponin T; smooth muscle actin; cardiomyocytes

challenging, however, due to tissue availability and sample quality. An additional concern for researchers is deciding the method of RNA extraction from samples. Typically, RNA is extracted from either whole biopsies or from isolated cardiac myocytes, each of which has advantages and disadvantages.

To determine gene expression in cardiac myocytes, many researchers use isolated cell methods for extracting RNA. One of these methods involves isolating live cardiac myocytes and individually picking them using a micromanipulator and micropipette for use in gene expression studies. This method can be useful and eliminates contamination of other cell types; however, gene expression could be altered by either the isolation technique (harsh digestions) or during the often lengthy time of cell culture. This method may also not yield accurate results for global gene expression changes since only a small number of cells are sampled. Since it has been shown that transcript levels differ between the areas of the heart (7, 19), comparing cells isolated from different areas may also be problematic.

Similarly, single myocytes can be selected via laser capture microdissection from tissue slices (12, 14). While this method is a precise way to obtain single, pure cells from a tissue section, it can be time consuming to collect the cells and the equipment is costly. Although the small number of collected cells may not be representative of the whole biopsy or organ, this method can be very beneficial if a specific population of cells, such as the area of infarction, is the basis of study.

Flow cytometry has also been used to sort cardiac myocytes for use in gene expression studies (6, 18). This method is very useful and can rapidly isolate a larger population of cells; however, it still relies on cardiac myocyte isolation procedures that may lead to gene expression changes. The use of flow cytometry is complicated by the need for specialized equipment and expertise and the need to establish conditions and markers to discriminate cell types and viability.

In contrast to the isolated cell method, quality total RNA or mRNA can easily and quickly be obtained from whole cardiac biopsies, using either commercially available kits or a modification of the phenol chloroform extraction method (3, 5, 8, 13). This is a common technique to extract RNA for gene expression studies of cardiac biopsies, and it does not require expensive equipment or specialized training, allowing most common laboratories to use this approach. Cardiac tissue, however, contains numerous cell types, and if a specific cell type is desired, these methods cannot discriminate the source of the RNA being extracted. Previous analyses with the human heart have demonstrated that cardiac myocytes only represent ~75–80% of the normal ventricular tissue volume and 25–30% of the total cell number (4, 15, 21). This high level of alternative cell types (mostly fibroblasts and smooth muscle cells) could

SINCE THE ADVANCEMENT of real-time PCR and microarray technology, there has been a great interest in determining the alterations in gene expression in human cardiac disease states (7, 9, 10). The ability to detect changes in the gene expression of receptors, signaling proteins, and muscle proteins and the reactivation of specific fetal genes have given great insight into the changes that occur during coronary ischemia, arrhythmias, cardiac hypertrophy, heart failure, and cardiac cell death (1–3, 9, 10, 13, 16). Human gene expression data collection is

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make the data interpretation of downstream applications, such as real-time RT-PCR, difficult.

Therefore, given some of the limitations associated with both isolated cell and whole biopsy procedures, we designed a unique cardiac myocyte purification method for RNA extraction. This procedure combines the benefits of using whole heart biopsies while adding modifications that increase selectivity for purifying cardiac myocytes which are normally associated with only cell isolation techniques. It is our hope that this method will increase the accuracy and ease of gene expression data collection from human heart biopsies and potentially other types of biopsies.

MATERIALS AND METHODS

Subjects

Eight total samples were obtained during transplant and routine cardiac operations at the Mid-America Heart Institute (St. Luke’s Hospital, Kansas City, MO). Five biopsies (2 donor left atrial appendages, 2 diseased left ventricular biopsies, and 1 diseased left atrial biopsy) were used for initial studies comparing purified versus non-purified techniques. Three additional biopsies (1 left atrial appendage and 2 right atrial appendages) were used as a subset for additional analysis of individual purification steps and RNA quality. Subjects were between the ages of 30 and 70 yr of age. Approval from St. Luke’s Hospital Institutional Review Board was obtained for this procedure, and all protocols were followed according to the Declaration of Helsinki. Biopsies were placed immediately in RNAlater (Ambion, Austin, TX) so as to preserve the RNA within the sample and then stored at 4°C.

Tissue Processing

All RNA extraction involving whole biopsy tissues used the RNasy fibrous tissue mini kit, whereas RNA extraction from cells after the various purification steps used the RNasy mini kit, both with DNase treatment (Qiagen; Valencia, CA). It was recommended by the manufacturer that no more than 30 mg of tissue be used so as not to exceed the RNA binding affinity of the column. Therefore, all samples were weighed and measured to be ~30 mg after removal of extraneous adipose or connective tissue.

All biopsies were cut into two pieces. One piece was used for whole biopsy RNA extraction (no purification, step 0) in which the biopsies were dissected into smaller pieces (~2 mm³) and homogenized with a rotor stator homogenizer (TissueMixer; Fisher Scientific, Pittsburgh, PA) in buffer RLT with β-mercaptoethanol, and instructions were followed according to the manufacturer. The other half of the biopsies went through the purification protocol where RNA was extracted at the end of the protocol as outlined in Purification Protocol. For the three additional biopsies, samples were taken at the end of each purification step (steps 1–3) for RNA extraction.

Purification Protocol

All of the following steps were performed in plates or tubes that were on ice so as to minimize RNA degradation.

Purification step 1. The tissue was dissected into small pieces (~2 mm³) while in RNAlater and quickly transferred to a 50-ml tube with 25 ml of Hank’s balanced salt solution (HBSS) that did not contain calcium or magnesium. HBSS was used as an isotonic solution to minimize the lysing of cells and the loss of RNA. Additionally, calcium and magnesium were excluded to minimize the divergent cation-mediated degradation of RNA. The small tissue pieces were homogenized at the lowest speed for 2 min until the solution was cloudy using a homogenizer that had been briefly rinsed in RNAlater. To separate the muscle tissue and cells from larger pieces of connective tissue, the homogenate was filtered through an 800-µm nylon mesh (Spectrum, Rancho Dominguez, CA) into a new 50-ml tube.

Purification step 2. This process was repeated using a 300-µm nylon mesh (Spectrum), and the filtered solution was then centrifuged at 2,000 g for 2 min. (Hermle Z300; Wehingen, Germany). Following the spin, the supernatant was removed and the pellet was resuspended in 5 ml of HBSS.

Purification step 3. This solution was filtered a final time through a 40-µm nylon cell strainer (BD Falcon, San Jose, CA). This final step trapped the myocytes on the filter while other debris flowed through and were discarded. The trapped myocytes were then washed off the filter using 25 ml of HBSS. The newly suspended myocyte solution was centrifuged at 2,000 g to pellet the cells for RNA extraction or resuspended in a smaller volume of 2 ml of HBSS for analyzing RNA location in myocytes.

Analysis of Total RNA from Myocytes

To determine the effectiveness of the purification procedure, we used both manual cell counting and RNA staining of the purified sample from three biopsies (1 diseased ventricle, 1 diseased atria, and 1 donor left atrial appendage). Acridine orange staining solution (1 µl; Invitrogen; Carlsbad, CA) was added to the 2 ml myocyte solution created in the last step of the purification procedure. The solution of cells was plated on glass dishes and incubated with acridine orange for 30 min. Ten random areas per plate were selected for analysis, and each area yielded ~40–60 objects. The myocytes were visually identified using a ×10 (UPLFLN 10×PH), ×40 (LUCPLFLN 40×PH), and ×60 (UPLFLN 60×OIPH) objective on the basis of striations and shape and were counted compared with objects that were unable to be identified (counted as unknown objects).

Fluorescence imaging was conducted using an Olympus IX51 inverted microscope (Center Valley, PA), Hamamatsu Orca-ERGA CCD camera (Bridgewater, NJ), Semrock Bright Line filter set (Rockester, NY), and X-cite 120 metal halide light source (EXFO, Mississauga, CA). When bound to DNA, acridine orange emits at 520 nm and was visualized with a FITC filter set, and when bound to RNA, the dye emits at 620 nm and was visualized with a Texas-red filter set. For each area analyzed per plate, a mask was created within SlideBook imaging software (Intelligent Imaging Innovations, Denver CO). This mask allowed for defining objects that emitted fluorescence and gave a fluorescence intensity value to each pixel. The exposure time and intensity was carefully chosen so that no pixels were saturated. The fluorescence values from the myocytes and unknown objects were then summed. Data were then added together for the 10 areas per plate, and a percentage of the total fluorescence from the myocytes compared with the total fluorescence from all other objects was calculated. The three different samples were then averaged.

Real-Time RT-PCR

Real-time RT-PCR was conducted on RNA extracted from samples that were used for both whole biopsy RNA extraction and the purification protocol. Real-time RT-PCR was performed using the TaqMan RNA to CT One-Step kit (Applied Biosystems; Foster City, CA) with a final reaction volume of 10 µl and using a Corbett Research Rotor Gene 6000 (Corbett Robotics, Sydney, Australia). The threshold levels were set above background fluorescence and in the exponential phase as previously described (20, 22, 23).

Primer-Probe Set

Primer-probe sets for peptidylprolyl isomerase A (PPIA; Hs00999994_m1), large ribosomal protein P0 (RPLP0; Hs00999902_m1), vimentin (Vim; Hs00185584-m1), α-smooth muscle actin (α-SMA; Hs00904949_m1), and troponin T (TNT; Hs00165960_m1) were purchased as 20× premade solutions from Applied Biosystems. The reaction efficiencies of the primer-probe sets were determined by using fivefold dilutions of human heart RNA similar to methods previously described (20, 22, 23).
**RESULTS**

The purification process is outlined in Fig. 1. Once practiced, the technique took ~12–15 min. There were no observable differences in the isolation procedure in terms of cardiac myocyte integrity and the ability to filter myocytes between atrial and ventricular samples or diseased and donor samples. The only difference observed in the data was higher cardiac TnT levels in the ventricular biopsy samples than in the left atrial and ventricular samples.

We determined the condition of myocytes and the localization of RNA after the purification protocol in three samples. After purification, most myocytes displayed normal morphology, exhibiting rod-like shape and defined striations (Fig. 2A). Acridine orange was used to visualize the location of DNA (Fig. 2B) and RNA (Fig. 2C) within the sample. DNA and RNA were found in and around defined nuclei in the intact myocytes as well as in myocytes that had been sheared during the homogenization step. Additionally, the background did not fluoresce, demonstrating the absence of free nucleic acids.

Acridine orange was also used to determine the amount of RNA attributed to myocytes versus other cell types after the purification protocol. The purity in the three samples was analyzed by both manual counting of Brightfield images and computer analysis of acridine orange-stained RNA. Brightfield cell analysis was conducted by counting the cells that expressed rod-shaped morphology and visible striations (representative cell in Fig. 2A). If a cell could not easily be visually identified as a myocyte, it was counted as an unknown. The unknown objects were most likely small, sheared myocytes, contaminating cell types, or small pieces of connective tissue. The total number of objects counted were 690, 394, and 517 for samples 1, 2, and 3, respectively. For the calculation of RNA in myocytes, the fluorescent intensity was measured for each object using the Texas-red filter. The largest fluorescence value for any pixel was 3,878, and the smallest value was 257. Since our camera displays a linear range of fluorescence up to 4,096 arbitrary fluorescent units, pixels were not saturated during the measurement of intensities. Manual counting indicated that 97.3 ± 1.5% of all objects were cardiac myocytes, and fluorescence imaging indicated that 98.4 ± 0.9% of all RNA was contained within the defined membranes of cardiac myocytes (n = 3).

To further verify that the purification technique reduced the amount of contaminating cell types, we used gene expression analysis of specific markers of cardiac myocytes, fibroblasts, and smooth muscle cells. Figure 3 displays real-time RT-PCR analysis comparing Vim, α-SMA, and cardiac TnT expression levels from RNA extracted from the eight biopsies in which one sample from each biopsy underwent purification and another sample from the same biopsy was not purified. Two different housekeeping genes were used for normalization so as to verify the results (Fig. 3A, PPIA; and Fig. 3B, RPLP0). The
reaction efficiencies were very similar for all primer/probe sets [90%, 93%, 91%, 89%, and 89% (R² >0.990) for PPIA, RPLP0, TnT, Vim, and α-SMA, respectively], and therefore 2-ΔCT could be used for analysis in this study. With the use of either housekeeping gene, the levels of TnT were higher and Vim and α-SMA were lower in the purified sample (P < 0.05). The data analyzed with PPIA and RPLP0 displayed similar fold changes in expression for each of the three genes of interest. When we compared the purified to nonpurified for TnT, Vim, and α-SMA, the fold change was 6.26, 0.48, and 0.51, respectively, using PPIA as the housekeeping gene, and 6.05, 0.38, and 0.45, respectively, using RPLP0.

RNA from various stages of the purification technique were used for real-time RT-PCR using PPIA as the housekeeping gene, and we obtained similar changes in expression. The largest increases in TnT were after steps 1 and 3. The largest decreases for Vim and α-SMA were both after step 3 (Fig. 4A). To demonstrate that the designed method did not substantially degrade RNA, the RIN of the extracted RNA from each purification step for the three samples was also determined. Figure 4B shows that the purification method did not significantly degrade the RNA (P > 0.05). For comparison purposes, the RIN of a sample of RNA from human heart total RNA purchased from Ambion was 7.4. The concentration of RNA obtained for steps 0, 1, 2, and 3 were 29.6 ± 9.2, 9.7 ± 1.9, 8.7 ± 3.2, and 6.5 ± 1.4 μg/ml, respectively (RNA was eluted in 100 μl total).

**DISCUSSION**

With advances in molecular biology combined with the use of human cardiac biopsy samples, the potential of translational cardiac research is very exciting. Nevertheless, there is still the issue of selecting the method for obtaining RNA from cardiac biopsies for downstream applications such as real-time RT-PCR. If RNA is extracted from the whole biopsy, then there are other contaminating cell types that contribute RNA to the total which may complicate the data interpretation. If cell isolation methods are used to obtain pure myocytes such as manual picking, laser capture microdissection, and flow cytometry,
then more specialized techniques and expensive equipment are needed. In addition, the gene expression may be altered during the procedure and more global changes in gene expression may be missed by analyzing individual cells. We have presented a new purification technique in this article that combines the advantages of simple whole tissue biopsy RNA extraction with the addition of steps to help purify the cardiac myocytes.

During the homogenization and purification process, we were concerned that cells could lyse and RNA would be lost, or that RNA from contaminating cell types would persist in solution, causing inaccurate data. To confirm that the extracted RNA from our purification protocol was from cardiac myocytes, fluorescent imaging was used to identify the source of RNA in our samples. While we did observe some sheering of myocytes due to homogenization, RNA was still visualized within the cytosol of even the sheared cells and was not visible in the cell solution. The RNAlater likely helped reduce RNA loss by preserving cell morphology and cell integrity. Therefore, these results demonstrate that cells isolated by this method maintain their structure and preserved genetic information and that there was no contaminating RNA free in the solution.

To further confirm that the majority of extracted RNA could be attributed to cardiac myocytes rather than contaminating cell types, calculations of the RNA intensity and area of fluorescence were computed after purification. On average, >98% of the RNA fluorescent intensity could be attributed to cardiac myocytes, which is in agreement with the results from visual cell counting in which we observed that >97% of all objects/cells were myocytes. As any objects that did not display visible striations or cardiac myocyte morphology were counted as unknown, these data are likely an underestimation of the amount of RNA contributed by myocytes, since some unidentified objects were likely myocyte fragments. Vliegen et al. (21) demonstrated that <30% of normal human left ventricular cells were cardiac myocytes, indicating our technique provides a significant purification of cardiac myocytes from a human biopsy.

The real-time RT-PCR results provided strong data supporting the purification of cardiac myocytes and the exclusion of contaminating cell types. We used three cell type-specific markers in our gene expression studies: cardiac TnT as the cardiac myocyte specific marker (17), Vim as the fibroblast marker (12), and α-SMA as the smooth muscle marker (11). The real-time RT-PCR results demonstrate a significant increase in TnT and a decrease in Vim and α-SMA mRNA from the purified compared with the nonpurified sample. It is likely that the residual expression of Vim and α-SMA is in part due to a low expression level of these genes by cardiac myocytes themselves. In a study using cardiac myocytes isolated by laser capture microdissection, there was still some expression of Vim by pure cardiac myocyte samples (12).

In a subset of samples, we analyzed gene expression during each major filtration step of the purification protocol. It was observed that the majority of filtration and purification occurred during steps 1 and 3. Specifically, step 3 was crucial for increasing the TnT expression and decreasing the Vim and α-SMA expression. This is not surprising since we purposely chose this filter size so as to be small enough to trap most cardiac myocytes but large enough to allow other cell types to flow through. Whereas the third filtration step was the most important in purification, filter steps 1 and 2 are still needed to help remove large debris that would clog the final filter step and lead to trapping of contaminating cell types.

We also tested the RNA quality during the purification procedure and did not observe any significant RNA degradation. The pretreatment of the sample with RNAlater, the rinsing of the homogenizer in RNAlater, and the short duration of the purification technique all likely contributed to the preservation of the RNA integrity. Since we did not observe significant RNA degradation during the purification, RNA quality is strongly dependent on the initial biopsy sample isolation and treatment. In that light, our starting RNA quality for our cardiac biopsies was similar to that of human cardiac RNA obtained from Ambion. Our gene expression studies and RNA quality analysis demonstrate the capability of this technique to successfully provide purified cardiac myocyte RNA that can be successfully used for downstream applications such as real-time RT-PCR.

![Graph](http://ajpheart.physiology.org/)

**Fig. 4.** A: gene expression of TnT, Vim, and α-SMA from samples taken during various filtration steps of the purification protocol as labeled in Fig. 1 (n = 3 biopsies). PPIA was used as the housekeeping gene for normalization. **Step 0** indicates RNA extracted from a piece of the same biopsy that underwent no purification. B: RNA integrity number (RIN) of RNA from each of the purification steps. RIN was measured using an Agilent Pico Eukaryotic Total RNA chip and an Agilent Bioanalyzer 2100 using the entire electrophoretic trace. There was no significant degradation of RNA during the purification protocol ($P > 0.05$; $n = 3$ biopsies). For comparison, RIN from a commercially available heart biopsy RNA was 7.4.
It is possible that the RNA obtained from this cardiac myocyte purification method could also be used for other applications such as microarray analysis, Northern blot analysis, and protein analysis. As little as 1 μg of RNA can be used for Northern blot analysis, and 100 ng can be used for microarray analysis. After the purification protocol, we obtained on average ~650 total ng of RNA from our 30-mg preparation. For Northern blot analysis, it would be recommended that more biopsy tissue be taken to ensure there is enough RNA. In the case of our specific human biopsy samples, the RIN values were high enough that the RNA could be used for microarray or Northern blot analysis. Microarray analysis would particularly benefit from this technique since it would be helpful to know the gene expression changes that are occurring in only the cardiac myocytes from a region of the heart. Since the particular focus of this study was on gene expression analysis, protein was not isolated from the samples. Nevertheless, we feel that this technique could be optimized to provide enough quality protein for proteomics approaches as well. This cardiac myocyte purification protocol could also be adapted for the purification of other cell types and use in various types of animal species and organs. Whereas the large size of cardiac myocytes makes them a good candidate for this type of filtration, other filter sets could be adapted for cells of different sizes.

Considerations for Successful Purification

First, the tissue should be kept in RNAlater until the last possible moment and small pieces should remain submerged in this protective liquid even while being cut. Once the pieces are removed from RNAlater, the entire purification scheme should be carried out as rapidly as possible. Tubes, pipets, filters, etc., should be prepared before starting, and the homogenizer could be sterilized and can be quickly rinsed in RNAlater to help prevent RNA degradation. If the RNA quality remains a concern in the subsequent steps, a solution such as a commercially available RNAse inhibitor could be added to all solutions following the removal from RNAlater.

Second, during this technique, rotor-stator homogenization was used to quickly dissect the tissue into smaller pieces in a small amount of time so as to minimize RNA degradation. The time of homogenization may change based on the type of homogenizer, and different speeds and times should be tested. If the homogenization is too vigorous, there may be an increase in sheared cells and debris. If the homogenization is too weak, then cells may not become dissociated and there will be a small amount of RNA collected in the end. Alternative methods of isolating cells such as using a mortar and pestle were not tried; however, this method typically requires the tissue to be frozen and pulverized, which may alter the shape/isolation of the myocytes and hinder the filtration process. Additionally, enzymes such as collagenase, papain, or elastase may be tried to help in the dissociation of cells. We used collagenase type II before and during homogenization in some preliminary experiments, but the collagenase digestion step added time to the protocol and we did not find that it significantly increased the myocyte yield. In these preliminary trials, it should be noted that collagenase was added to our cold solutions, which undoubtedly affected its efficiency. Heating the collagenase solutions to its optimal temperature of 37°C would increase digestion, but the increase in temperature may also lead to degradation of RNA.

Third, during filtration, attention should be paid to running the sample through a clean area of the filter. If the filter in the first two steps becomes even slightly congested, it is likely that some cardiac myocytes will become trapped in the large debris and discarded. Therefore, a large area of filter should be used and a new filter should be used for each experiment.

In summary, this proposed method provides researchers with a simple and quick procedure to purify cardiac myocytes using human cardiac biopsies, allowing for more accurate gene expression results. The method requires only basic laboratory materials and a short amount of time, and, therefore, most common clinical and basic research laboratories would be able to carry out this technique.

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