Technique for quantitative RT-PCR analysis directly from single muscle fibers

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Wacker MJ, Tehel MM, Gallagher PM. Technique for quantitative RT-PCR analysis directly from single muscle fibers. J Appl Physiol 105: 308–315, 2008. First published May 8, 2008; doi:10.1152/japplphysiol.00897.2007.—The use of single-cell quantitative RT-PCR has greatly aided the study of gene expression in fields such as muscle physiology. For this study, we hypothesized that single muscle fibers from a biopsy can be placed directly into the reverse transcription buffer and that gene expression data can be obtained without having to first extract the RNA. To test this hypothesis, biopsies were taken from the vastus lateralis of five male subjects. Single muscle fibers were isolated and underwent RNA extraction, biopsies were taken from the vastus lateralis of five male subjects. Single muscle fibers were isolated and underwent RNA extraction technique 1 or placed directly into reverse transcription buffer technique 2. After cDNA conversion, individual fiber cDNA was pooled and quantitative PCR was performed using primer-probes for β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, insulin-like growth factor I receptor, and glucose transporter subtype 4. The no RNA extraction method provided similar quantitative PCR data as that of the RNA extraction method. A third technique was also tested in which we used one-quarter of an individual fiber’s cDNA for PCR (not pooled) and the average coefficient of variation between fibers was ≤8% (cycle threshold value) for all genes studied. The no RNA extraction technique was tested on isolated muscle fibers using a gene known to increase after exercise (pyruvate dehydrogenase kinase 4). We observed a 13.9-fold change in expression after resistance exercise, which is consistent with what has been previously observed. These results demonstrate a successful method for gene expression analysis directly from single muscle fibers.

skeletal muscle; gene expression; glucose transporter subtype 4; insulin-like growth factor I receptor; quantitative reverse transcription-polymerase chain reaction; pyruvate dehydrogenase kinase-4

The use of quantitative reverse transcription (RT)-PCR has greatly enhanced the ability of researchers to analyze changes in gene expression in various animal and human experiments. While analysis of gene expression is commonly measured from mRNA extracted from tissue, more recent approaches have employed the use of RT-PCR to analyze gene expression at the single-cell level. Single-cell studies eliminate the complexity of mRNA analysis of whole tissue, which unavoidably contains numerous cell types and can add difficulty to data interpretation. The ability to discern changes of mRNA at the single-cell level has become an important tool in muscle physiology for not only discriminating muscle fiber types but also analyzing gene expression differences between fiber types.

Early work with the technique of single-fiber RT-PCR involved determining myosin heavy chain (MHC) isoform composition. One group of researchers used a technique in which fiber fragments were microdissected, and then RNA extraction from single fibers was performed using a modified oil-well technique (14) to protect the small volume solution from evaporation and contact with CO2 (16, 17, 23, 26). Fibers were put into a high-salt extraction medium under mineral oil with RNase inhibitors. The samples were then incubated for 60 min at 4°C to allow time for RNA extraction. Primers, dNTPs, and reverse transcriptase were then added in a dilution medium to create optimal conditions for the reverse transcriptase step. Another group of researchers (4, 8, 15) analyzed MHC isoforms utilizing RNA that was extracted from single fibers based off of the well-documented method by Chomczynski and Sacchi (4). Both of these methods were successfully employed by these groups for end-point RT-PCR analysis of MHC isoform types.

More recent research has employed the use of quantitative RT-PCR and involves analysis of expression of not only MHC isoforms but also various genes involved in cell processes and signaling pathways (1, 3, 12, 22, 31). The methodologies for quantitative RT-PCR involve similar fiber-dissection techniques, but typically fibers are individually placed in an extraction buffer (or RNA protection buffer), and the RNA is then extracted from these fibers using commercially available kits or reagents (1, 3, 12, 22, 31). A RT step is then employed to convert the mRNA to cDNA. The cDNA from the same muscle fiber types or groups is then either combined to run quantitative PCR reactions with genes of interest or run separately to analyze expression of genes. A housekeeping gene (with stable expression levels despite treatments) is usually selected to normalize the data for comparison between treatment groups.

In this paper, we present a novel technique for quantitative RT-PCR in which gene expression can be analyzed from single fibers without prior RNA extraction protocols or additional steps to purify the RNA. In this technique, individual muscle fibers are dissected from a muscle biopsy and placed directly into buffer for the RT step. This technique was tested using two commonly used housekeeping genes, β2-microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as two genes that are involved in skeletal muscle growth and metabolism, insulin-like growth factor (IGF) I receptor (IGF1R), and glucose transporter subtype 4 (GLUT4). In this study, we were able to demonstrate that this technique provides similar data to the tested RNA extraction protocol method, which saves valuable time and expense.
METHODS

Subjects. Muscle biopsies from the vastus lateralis were obtained from 10 healthy, nonobese, male subjects between the ages of 18 and 22 yr. The surgical procedure was approved by the Institutional Review Board at the University of Kansas, and subject consent was obtained as per the guidelines set by the Declaration of Helsinki. The biopsy samples were immediately placed in RNALater (Qiagen) and stored at −20°C.

Single-fiber isolation. Small sections of each of the muscle bundles were separated while in RNALater solution. The separated muscle section was then placed in phosphate-buffered saline, and single muscle fibers were then isolated from the muscle bundle using fine tweezers and a stereomicroscope (Bausch and Lomb). The fibers were freed of connective tissue and other debris and quickly placed into either RNA extraction buffer or reverse transcription buffer.

RT. The Superscript III (SSIII) two-step quantitative RT-PCR kit (Invitrogen, Carlsbad, CA) was utilized for all RT reactions. This kit utilizes a proprietary mixture of random hexamer and oligo(dT) primers for the RT reaction. RT reactions were incubated at 25°C for 10 min, followed by 55°C for 30 min in a PTC 200 thermocycler (MJResearch, Waltham, MA).

Quantitative PCR. Quantitative PCR reactions were set up using an automated liquid handling system (model CAS-1200, Corbett Robotics, Sydney, Australia) and quantitative PCR was performed using the Corbett-Research Rotor Gene 3000 (Corbett Robotics). The threshold line for determining cycle threshold (CT) values was set at the first indication of amplification above the background noise in the exponential phase of the curve. Because all of the runs were very similar, the threshold was able to be held constant between runs. The threshold was always in the exponential phase and above any background fluorescence.

Primer-probe sets. Primer-probe sets for GAPDH (Hs99999905_m1), B2M (Hs99999907_m1), IGF1R (Hs00609566_m1), GLUT4 (Hs00168966_m1), and pyruvate dehydrogenase kinase 4 (PDK4) (Hs00176875_m1) were obtained as premade 20× mixtures from Applied Biosystems (Foster City, CA). The primer-probe sets are designed utilizing TaqMan chemistry and are optimized to yield 100% efficiency. The exact sequences are proprietary information, but for all of the tested genes the primers span an exon-exon junction and for IGF1R and GLUT4 the probe also spans an exon-exon junction.

Reaction efficiency. All primer sets were tested to determine what the reaction efficiencies were under experimental conditions. Reaction efficiencies were determined using cDNA converted from RNA that was isolated from a muscle biopsy, using technique 1, and using technique 2. RNA was extracted from the whole muscle biopsy using the Qiagen RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). RNA was eluted in a final volume of 50 µl of RNase- and DNase-free water. For the RT step, 8 µl of purified RNA was combined with 10 µl of RT buffer and 2 µl of the SSIII reverse transcriptase (for a total of 20 µl). After RT, 5 µl of cDNA was then combined with 10 µl of 2× Platinum Taq qPCR supermix reaction buffer, 1 µl of MgCl2 (final concentration of 5.35 mM), 1 µl of the 20× primer-probe mixture, and 3 µl of water. Four 10-fold dilutions were then made from this stock solution using the robotic arm to generate a standard curve. To calculate reaction efficiencies using techniques 1 and 2, cDNA was first prepared using protocols outlined in techniques 1 and 2 listed below, and twofold dilutions were made to construct a standard curve. Twofold dilutions were utilized because the concentration of cDNA obtained from single cells is much lower compared with whole muscle and gene expression is already at a high CT value for some genes. For calculation of reaction efficiency, the log of the RNA concentration was plotted on the x-axis and CT values on the y-axis. A line of best fit was generated, and reaction efficiencies were determined using the following equation: reaction efficiency = [10(−1/10m)]/2·100, where m is the slope of the line. Each data point was tested in triplicate and R² values were calculated.

Technique 1. (Fig. 1). Twenty-five individual muscle fibers from five subjects were isolated and placed in RNA extraction buffer, and the RNA was extracted from the combined fibers using a final volume of 50 µl. The RT step and PCR were carried out as previously stated for testing reaction efficiency. By using these concentrations, the amount of cDNA used per PCR reaction was approximately equivalent to the cDNA from one cell (25 fibers in 50 µl final RNA volume = 1 fiber/µl; 8 µl of RNA was used in a total volume of 20 µl RT reaction which equals 4 fibers/20 µl or 1 fiber/5 µl; 5 µl was used in the PCR reaction, which is approximately equal to 1 fiber).

Technique 2. Twenty-two individual fibers were isolated and placed directly into separate tubes each containing 2.5 µl of the RT master mix buffer, 0.5 µl SSIII, and 2 µl RNase- and DNase-free water (total of 5 µl). After the RT reaction, the cDNA products were combined into one tube. Five microliters of cDNA was then used for the quantitative PCR reaction (5 µl of cDNA is equal to the total amount of RT reaction mix for 1 fiber). A control experiment was also performed in which a muscle biopsy was taken, and half of the biopsy was placed in RNALater and half was placed in liquid nitrogen. Fibers were picked from both halves of the sample using this protocol and tested using the GAPDH primer-probe set.

Technique 3. Twenty-two individual fibers from each subject were isolated and placed directly into separate tubes each containing 10 µl of the RT master mix buffer, 2 µl of SSIII, and 8 µl RNase- and DNase-free water. After the RT reaction, 5 µl of the cDNA for each individual fiber was then used for each quantitative PCR reaction (one-quarter of the total RT reaction for a fiber). This technique is different from technique 2 in that the cDNA from each fiber was not pooled and PCR was run separately for each fiber using only one-quarter of each muscle fiber’s cDNA for each reaction.

Controls. Two control tubes were also run with each subject for each of the three techniques. One control tube was run exactly the same for each technique except that a muscle fiber was not placed into the buffers (no template control tube). Another control tube was run the same in all the techniques (including adding the muscle fiber) except that the RT enzyme was not included into the RT mixture (no RT control tube).

Data analysis of the three techniques. cDNA from techniques 1 and 2 were run in triplicate and averaged and presented with standard deviation. A Student’s unpaired t-test was utilized to compare differences in CT values between techniques 1 and 2. Technique 3 was not included in statistical analysis because the amount of cDNA that was used was not the same as techniques 1 and 2. Average CT was calculated for technique 3 on the basis of all fibers tested for that subject, and the coefficient of variation (CV = SD/mean·100) was calculated for each subject and then averaged.

Detection of differences in gene expression. Five recreationally active subjects were utilized for this study to verify that the no RNA extraction technique would detect changes in gene expression. Gene expression differences were measured for pyruvate dehydrogenase kinase 4 (PDK4), which has been previously shown to be upregulated after resistance exercise (20, 21, 25, 30, 31). Methodology utilized for this study was similar to that of two other previous studies for comparison purposes (30, 31). Biopsies from the vastus lateralis were taken 30 min before and 4 h after a bout of resistance exercise. The resistance exercise consisted of leg extensions of 2 sets of 10 repetitions and 1 set to failure of 80% of the subject’s maximum lift capacity. Subjects did not exercise 48 h before the experiment and were fed a controlled diet for 24 h during the experimentation period. Muscle fibers were isolated from the biopsies, and protocols were followed as listed in technique 2. PDK4 CT values were normalized to GAPDH (ΔCT) because it has been previously shown that GAPDH is a stable housekeeping gene to use for this type of exercise (30, 31) and for comparison purposes to these other studies. Fold changes in gene expression were calculated using 2−ΔΔCT comparing pre- and postexercise numbers. Values were then averaged and SE of the mean.
calculated. A Student’s t-test was used to determine statistical difference using the linear numerical values (P < 0.05).

RESULTS

Four different genes were analyzed in this study using three different techniques. The three different techniques of single muscle fiber quantitative RT-PCR utilized in this study are outlined in Fig. 1. Technique 1 utilized a RNA extraction procedure on isolated fibers before the RT step, whereas techniques 2 and 3 placed individual fibers into the RT buffer and the RT step was directly run. Technique 2 combined the cDNA from fibers into one pool of cDNA for PCR, whereas in technique 3 PCR was carried out on one-quarter of the cDNA extracted from each individual muscle fiber.

Figure 2 displays the raw quantitative PCR results from one subject using all three techniques. Because the cDNA was pooled for both techniques 1 and 2, these PCR reactions for each gene were run in triplicate, and the average CT (±SD) is reported in Fig. 3. Statistical analysis indicated that there were no differences between the CT values of the housekeeping genes, GAPDH (P = 0.123) and B2M (P = 0.558), between these methods, but there was a difference in CT values for GLUT4 (P = 0.001) and IGF1R (P = 0.028) with technique 2 having a lower expression level.

To confirm that the general protocol for these techniques did not yield low reaction efficiencies, all primer-probes sets were tested from standard dilutions of cDNA created using RNA from a muscle biopsy, technique 1, and technique 2. The reaction efficiencies for the RNA extracted from the homogenized muscle biopsy and from the isolated muscle fibers used in techniques 1 and technique 2 were as follows: GAPDH: 97%, 96%, 95%; B2M: 100%, 102%, 102%; IGF1R: 97%, 102%, 97%; GLUT4: 95%, 100%, 97%; PDK4: 102%, 100%, 97%, respectively. R² values for all primer-probe sets were >0.992, 0.95, and 0.94 for the muscle biopsy, technique 1, and technique 2, respectively. Results for GAPDH and B2M were similar to that found in a previous study (27).

We also wanted to determine whether fibers preserved in RNAlater would yield different results than fibers that were frozen in liquid nitrogen. Therefore, half of a muscle biopsy was placed in liquid nitrogen and the other half in RNAlater. The real-time curves for the fibers isolated from liquid nitrogen and RNAlater mirrored each other in shape and slope, but the liquid nitrogen fibers displayed a higher CT value. The CT values comparing fibers isolated from liquid nitrogen and RNAlater were 2.44 and 2.47 for GAPDH and B2M, respectively.

We included an additional technique that used a slightly different approach than technique 2. Technique 3 measured amplification from individual fibers instead of pooled fibers. Therefore, the success rate and variability of the expression of individual fibers were analyzed. There was successful amplification in 89% of all fibers tested using this technique. In most instances if there was no amplification in one of the primer-probe samples then there was no or low expression levels in the other primer-probe samples. If there was no expression in any of the primer-probe sets then the fiber was discarded from the data set. CV of the CT value was calculated for each group of...
collected fibers per subject. The CV values ranged from 2.5 to 7.8 for all genes tested. The average CVs for the five subjects for GAPDH, B2M, GLUT4, and IGF1R are displayed in Fig. 4.

There was no amplification in the no template or no RT control tubes for any of the techniques for IGF1R or GLUT4 primer-probe sets. However, there was amplification for the B2M primer-probe set in the no template control in techniques 1 (5/5 subjects), 2 (2/5 subjects), and 3 (1/5 subjects) with an average CT of 31.5 ± 1.1, 32.2 ± 0.6, and 30.8, respectively. Amplification also occurred in the no RT control with B2M in techniques 2 (5/5 subjects) and 3 (3/5 subjects) with average CT values of 33.0 ± 1.1 and 32.2 ± 1.9, respectively. GAPDH amplification occurred in the no RT control in techniques 1 (1/5 subjects), 2 (4/5 subjects), and 3 (2/5 subjects) with average CT values of 36.5, 38.5 ± 1.8, and 34.0 ± 0.1, respectively.

Fig. 3. Graph comparing cycle threshold values for each of the 4 genes tested using the 3 different techniques outlined in Fig. 1. *P < 0.05 comparing techniques 1 and 2 cycle threshold values. Technique 3 only used one-quarter of the cDNA from each cell and therefore was not used for comparison in statistical analysis.

Fig. 4. Graph of the average coefficient of variation of the cycle threshold values for 5 subjects with each gene using technique 3. Twenty-two fibers were tested for each of the 5 subjects, and the coefficient of variation was then averaged. P > 0.05 comparing the genes.
Innovative Methodology

To verify that this technique does in fact measure differences in gene expression, the levels of PDK4 were measured using technique 2 in muscle fibers before and after a bout of resistance exercise. Values for the five subjects’ biopsies were normalized using the housekeeping gene GAPDH, and then fold changes were calculated using the $2^{-\Delta \Delta CT}$ method to compare pre- and postexercise values for each subject. Figure 5 demonstrates that a 13.9 ± 5.1 fold change in gene expression of PDK4 was detected comparing pre- and 4 h postexercise expression levels ($P < 0.05$).

DISCUSSION

Gene expression analysis of single muscle fibers is a common technique used in muscle physiology. For this study, we hypothesized that gene expression data could be obtained directly from isolated individual muscle fibers without having to first isolate the RNA. The use of this technique would save time, effort, and money in obtaining muscle fiber gene expression data. To test this hypothesis, we used quantitative RT-PCR analysis of four genes with muscle fibers obtained from five different biopsies utilizing both a RNA extraction method (technique 1) and a method that did not include an RNA extraction step (technique 2).

Techniques 1 and 2. Our results indicate that both methods provided consistent CT values and low SD values. In comparison between techniques 1 and 2, there were no statistical differences in values between GAPDH and B2M, although the no RNA extraction average CT values were slightly higher. Typically, some mRNA is lost in RNA purification methods; therefore, it is likely that not all of the mRNA was able to escape from the fiber in the no purification method because the CT values were similar between the two methods. There was a statistically higher CT value with the no RNA extraction method for IGF1R and GLUT4. These differences are likely not due to alterations in reaction efficiency because all genes for these techniques were close to 100% in efficiency using techniques 1 or 2. The higher CT value may indicate that more mRNA is available to the RT enzyme in the RNA extraction method for these genes. It is also possible that the efficiency of converting the mRNA to cDNA may be slightly better in the RNA purified method especially in cases of genes of lower expression levels than housekeeping genes.

Our findings that the RT step can occur without prior RNA purification of the muscle fibers are not unexpected. Members of our research group as well as other laboratories have successfully used a similar technique for single-cell RT-PCR to analyze gene expression in cultured neurons (5, 19, 24, 28). In work with nodose ganglion, we were able to isolate individual cultured neurons using a microscope and glass micropipette attached to a micromanipulator, and the cells were placed directly into RT reaction buffer for successful gene expression analysis (28). It is likely that the initial heating stage of the RT step (55°C) damages the cell enough that the mRNA is able to escape the cell and become available to the RT enzyme.

Technique 3. Whereas technique 2 utilized cDNA that was pooled from multiple individual fibers, technique 3 utilized the no RNA extraction method using a fraction of a single muscle fiber’s cDNA. The rational for this series of experiments is that some investigators may want to analyze multiple genes from one individual muscle fiber. Although this technique may seem challenging because there is little cDNA obtained from a single cell and this cDNA is further divided between primer-probe sets, we believed that it was important to determine whether this approach would work. This technique would be beneficial because it allows one to study multiple gene expression analysis at the single-cell level without pooling multiple fibers together (which may be heterogeneous in the gene makeup, fiber type, etc.). Being able to analyze multiple genes from the same fiber (by using only part of the cDNA for each primer-probe set) would allow for the use of a housekeeping gene and multiple genes of interest in the expression analysis. Therefore, one-quarter of the cDNA created from one fiber was used for each PCR reaction. In these experiments, there was an 89% success rate for successful amplification from isolated fibers. Interestingly, for our laboratory’s previous experimental protocol with neurons we obtained a similar success rate (89%) (28). The lack of amplification in some tubes was most likely due to failure in successfully transferring a fiber to the buffer. It is also possible that there were pipetting errors or other technical problems associated with a reaction. For those reactions that did successfully display an amplification product, there was surprisingly low variation in CT values. The largest average CV for a subject using technique 3 was <8%. This seems to indicate that consistent amplification between muscle fibers is possible using this method. Successful use of this technique demonstrates that multiple genes can be analyzed directly from one fiber for comparative analysis at the single-cell level.

GAPDH, B2M, IGF1R, and GLUT4. For testing these various techniques in this study, we purposely selected two housekeeping genes as well as two muscle protein genes. The two highly expressed genes, GAPDH and B2M, were selected because they are both commonly used as housekeeping genes for comparative quantitative PCR studies. We obtained consistent CT values in all techniques with low CV for both these genes in our study.

The rationale for selecting the two muscle protein genes, IGF1R and GLUT4, for use in this study was twofold. For the first reason, these genes are lower expressed than the housekeeping genes and therefore would be beneficial to test with this method. For the second reason, they are important in the treatment...
study of muscle physiology and metabolism and are good candidate genes for future study. Specifically, IGFs and their isoforms, binding proteins, and receptors have been well documented to play an important role in muscle growth and development (9, 18). IGF1- and IGF1R-related genes have been measured in recent studies using quantitative RT-PCR to determine expression level changes as related to hypertrophy and sarcopenia (2, 7, 13, 29). Most of the work with IGF has been done with expression at the tissue level and would be an excellent candidate to be further explored at the single fiber level using the present technique. GLUT4 is the primary sugar transporter in skeletal muscle and plays an important role in glucose homeostasis in the body. There are many factors that can influence the expression of GLUT4 in skeletal muscle, such as insulin, thyroid hormone, exercise, neural innervation, and atrophy (11). Conditions that alter the expression of GLUT4 are particularly important in glucose tolerance and homeostasis in the body. Immunolocalization techniques have primarily been used to analyze GLUT4 expression in skeletal muscle fibers (6, 10); however, the use of single-cell quantitative RT-PCR could be of great use in determining the specific controlling mechanisms regulating GLUT4 expression.

Successful detection of gene expression changes. To ensure that the use of the no RNA removal technique could be used to detect changes in gene expression, we tested a gene (PDK4), using technique 2, that has been previously documented to be altered during exercise. We used methods similar to that of two other recently published studies using PDK4 (30, 31). Similar to these previous studies, biopsies were taken before and after a bout of resistance exercise, and levels of PDK4 were detected using real-time RT-PCR normalized to GAPDH. GAPDH was chosen as the reference gene to compare our findings with these other studies and also because these authors have previously demonstrated that GAPDH expression is not altered with this type of exercise (30, 31). In addition, another study has also recently shown that GAPDH is the most stable housekeeping gene during and after an incremental treadmill run to volitional exhaustion (12).

We observed a 13.9-fold change in gene expression of PDK4 after exercise, which is similar to findings by other investigators (20, 21, 25, 30, 31). In one study using RNA extracted from whole muscle, it was observed that there was an 8 fold change in PDK4 expression 4 h after exhaustive one-legged knee extension exercise at 70% maximal resistance after the fifth consecutive day of exercise (21). Yang et al. (30) also utilized RNA extracted from whole muscle biopsies in active subjects and observed a 26-fold increase of PDK4 at 4 h after undergoing 3 × 10 bilateral knee extensions at 70% maximum. Yang and colleagues also conducted a study that demonstrated PDK4 expression changes in isolated type I and IIa muscle fibers in sedentary men who underwent 3 × 10 bilateral knee extensions at 65% maximum. Interestingly, they observed approximately a onefold change for type I and a twofold change for type II fibers. We observed much larger changes in PDK4 at the single-fiber level, and therefore it might be possible that the no RNA extraction method may be more sensitive at detecting gene expression changes at the single-fiber level. However, our study utilized active men vs. sedentary men and 80% maximum repetitions vs. 65%, and thus variability between these two studies most likely accounts for the difference. In summary, we were able to use this technique to successfully detect changes in expression of a gene that has previously been shown to be upregulated during exercise.

Potential limitations and precautions for this technique. As stated earlier, there may be a difference between the no RNA extraction method and the RNA extraction method in detection of genes of lower expression. One issue would be that if the gene of interest is at an extremely low expression level, the RNA extraction method may be a more sensitive method to detect the gene. Another issue is that it is possible that there could be a difference in mRNA availability or conversion of mRNA to cDNA in low-abundant messages between the methods. In general, this would likely not be a problem as long as the comparative quantitative RT-PCR is used, samples are all treated consistently, and reaction efficiency is similar for all genes. However, it could be argued that this may be problematic if one gene is markedly upregulated between treatments. We believe that this difference in expression would have to be extreme between treatment groups since we tested a gene that is highly upregulated during exercise (PDK4) and has an expression level similar to IGF1R and GLUT4, and we obtained results that were similar to other reports that had used the RNA extraction method (20, 21, 25, 30, 31).

With whatever genes of interest are chosen for single-fiber quantitative RT-PCR, care needs to be taken to avoid amplification of genomic DNA or contaminating RNA using this technique. It is possible that the genomic DNA from the nuclei from each skeletal muscle fiber or from other contamination may become amplified during PCR. We have tested all five genes for skeletal muscle samples using controls that do not contain reverse transcriptase as well as controls that do not contain a template. Only B2M and GAPDH had any expression that occurred in these controls, and the expression levels were well below that of the normal samples (>9 cycles difference for B2M and >14 cycles difference than normal samples for GAPDH for the no RT control; greater than 8 cycles difference than normal samples for B2M for the no template).

For the no-RT controls it is possible that amplification occurred from either amplification of pseudogenes or detection of genomic DNA. For example, GAPDH is known to contain pseudogenes as part of the human genome which do not contain introns and therefore may be amplified. Although the exact sequences of our primer-probe sets were proprietary, we know that the B2M and GAPDH sets utilized primers that did span an exon-exon junction, but the probe did not split this junction. Therefore, it is possible that some detection of genomic DNA by the B2M and GAPDH primer-probe sets occurred in the no RT controls but at a much reduced efficiency (because the probe may be cleaved by amplification off of 1 primer). The amplification that was observed in this study was small because a 9-cycle difference between B2M samples and controls and a 14-cycle difference between GAPDH samples and controls would indicate a 512- and 16,284-fold difference in expression, respectively. This indicates that the amount in the control tubes is extremely small compared with the sample tubes would not alter experimental data. However, by designing primers and probes that span an exon-exon junction this problem, can be further minimized or eliminated. This can be observed in the primer-probe sets for IGF1R, GLUT4, and PDK4, which utilized probes that spanned an exon-exon junction and did not demonstrate amplification in the control tubes.
For the no template controls, it is possible that there was some contamination of DNA or mRNA in the reaction mixture or tubes. However, it is unusual that we did not observe amplification in the no template control tubes for any of the other genes. Interestingly, we observed amplification with only B2M in the no template control in a previous study utilizing RNA from whole muscle (27). This indicates that the observed amplification is not due to the technical aspects of this particular study with single fibers, but may be related to the specific primer-probe set. Although any form of amplification in control tubes is unwanted, if there is amplification, a large CT difference between the samples and the controls is desired. For example, an 8-cycle difference that we observed would indicate a 256-fold expression difference between the sample and the control if there is a doubling of DNA in each PCR cycle.

In addition to concerns of amplification of DNA, it is also possible that the use of RNAlater may alter the results using this technique since fibers are placed directly in the RT mix. RNAlater is a liquid that is commonly used in gene expression studies to protect cells and tissues from RNA degradation during storage. It has many advantages over liquid nitrogen, which includes being able to handle tissue at room temperature and easier storage. In our experience, it is also much easier to isolate single muscle fibers stored in RNAlater because they are more firm than those from liquid nitrogen. Fibers stored in liquid nitrogen become limp and are more easily shredded and torn. However, to determine whether RNAlater pretreatment may alter the results, fibers were isolated from a biopsy in which half the biopsy was placed in liquid nitrogen and half in RNAlater. Interestingly, there was a higher CT value obtained from the RNAlater sample for both GAPDH and B2M (2.44 CT values respectively comparing liquid nitrogen /H9004 vs. RNAlater. Interestingly, there was a higher CT value obtained comparing liquid nitrogen /H9004 vs. RNAlater). We believe that the higher integrity of the fibers from the RNAlater may have actually hindered the mRNA “leaking” out of the cell when placed in the RT buffer and therefore may affect the RT reaction and quantity of cDNA produced in this technique. We do not believe that the RNAlater in the RT mix directly affected the reaction because the fibers are isolated and washed in saline and because the real-time curves for liquid nitrogen and RNAlater fibers mirrored each other in slope and shape. However, the use of RNAlater and potential effects should be taken into consideration by the researcher before deciding on the best storage method.

Another potential concern in this method would be the large percentage of RT reaction mixture carried over into the PCR reaction used in our protocol. We do not believe that this affected the reaction efficiencies of our specific primer-probe sets because this same percent was used to create the standard curve for reaction efficiency calculations, and our first point of the standard curve (which had the RT mixture carryover) was not significantly deviated from the line of best fit for calculating reaction efficiencies. However, this is another consideration that should be noted by the researcher. If RT reaction mix carryover is a concern, then a lower RT volume or higher PCR volume could be used to reduce the percentage of RT reaction mix carryover.

Conclusion. In conclusion, in this study we have shown that direct RT without RNA purification can yield consistent results. There are several benefits to this technique which include no additional time spent in conducting RNA extraction protocols, no extra expense of reagents and kits for extracting RNA, and less handling of the cells and RNA and therefore less chance for contamination in the samples or loss of sample. We have also demonstrated that gene expression can be obtained from a portion of a single fiber’s mRNA and therefore multiple genes can be analyzed using one fiber. Thus this technique may provide researchers with another helpful tool for skeletal muscle gene expression analysis.

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