Effects of creatine supplementation on housekeeping genes in human skeletal muscle using real-time RT-PCR

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Murphy, R. M., K. K. O. Watt, D. Cameron-Smith, C. J. Gibbons, and R. J. Snow. Effects of creatine supplementation on housekeeping genes in human skeletal muscle using real-time RT-PCR. Physiol Genomics 12: 163–174, 2003. First published November 5, 2002; 10.1152/physiolgenomics.00060.2002.—The present study examined the validity and reliability of measuring the expression of various genes in human skeletal muscle using quantitative real-time RT-PCR on a GeneAmp 5700 sequence detection system with SYBR Green 1 chemistry. In addition, the validity of using some of these genes as endogenous controls (i.e., housekeeping genes) when human skeletal muscle was exposed to elevated total creatine levels and exercise was also examined. For all except 28S, linear relationships between the logarithm of the starting RNA concentrations and the cycle threshold ($C_T$) values were established for $\beta$-actin, $\beta$2-microglobulin ($\beta$2M), cyclophilin (Cyc), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We found a linear response between $C_T$ values and the logarithm of a given amount of starting cDNA for all the genes tested. The overall intra-assay coefficient of variance for these genes was 1.3% and 21% for raw $C_T$ values and the linear value of $2^{-C_T}$, respectively. Interassay variability was 2.3% for raw $C_T$ values and 34% for the linear value of $2^{-C_T}$. We also examined the expression of various housekeeping genes in human skeletal muscle at days 0, 1, and 5 following oral supplementation with either creatine or a placebo employing a double-blind crossover study design. Treatments were separated by a 5-wk washout period. Immediately following each muscle sampling, subjects performed two 30-s all-out bouts on a cycle ergometer. Creatine supplementation increased ($P < 0.05$) muscle total creatine content above placebo levels; however, there were no changes ($P > 0.05$) in $C_T$ values across the supplementation periods for any of the genes. Nevertheless, 95% confidence intervals showed that GAPDH was variable, whereas $\beta$-actin, $\beta$2M, and Cyc were the least varying genes. Normalization of the data to these housekeeping genes revealed variable behavior for $\beta$2M with more stable expressions for both $\beta$-actin and Cyc. We conclude that, using real-time RT-PCR, $\beta$-actin or Cyc may be used as housekeeping genes to study gene expression in human muscle in experiments employing short-term creatine supplementation combined with high-intensity exercise.

gene expression; quantitative RT-PCR; endogenous controls; biological variability; PCR efficiency

The employment of the reverse transcription polymerase chain reaction (RT-PCR) method has been widely used for the analyses of gene expression in a number of systems, including skeletal muscle. Over recent years the development of “real-time” RT-PCR equipment has introduced a technique with higher throughput and more quantitative capabilities. As with other methods such as Northern hybridization and ribonuclease (RNase) protection, real-time RT-PCR quantifies the relative abundance of the gene of interest against a constitutively (unregulated and constant), and often highly expressed gene, or a gene which has been shown to vary little across an intervention, referred to as an endogenous control or housekeeping gene. In RT-PCR, housekeeping genes are active references that are used to standardize between amounts of samples assayed. As such, their constitutive, or least changing, nature must be determined for the given experimental conditions. Commonly used housekeeping genes for studies involving mRNA measurements in human skeletal muscle include 18S (10, 12) and 28S ribosomal RNA (rRNA) (29), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (31). It has become apparent that there is no single housekeeping gene that is suitable for all experimental conditions, and a number of papers have been published looking at the viability of using certain genes as housekeeping genes in different systems using various methodologies (11, 13, 14, 27, 28, 33, 34).

Dietary creatine supplementation (CrS) is a widely used intervention in exercise science and, more recently, in clinical studies examining various muscular disease states (for review, see Ref. 32). Many studies have demonstrated that CrS results in an elevated total creatine content in skeletal muscle (9, 20, 26). Importantly, this increase may induce changes in mus-
ucle gene expression perhaps by altering the energy state of the cell or by cell volume-mediated processes. For example, changes in both gene and/or protein expression have been recently reported in models involving CrS and resistance training compared with a placebo group (15, 31). In studies examining gene expression, knowledge of the variations in the expression of housekeeping genes under given experimental conditions is required for valid data interpretation. Although real-time RT-PCR requires the use of a housekeeping gene for interpretation of the data, the relative constitutive nature of this gene must first be established, as must the use of raw data values obtained in real-time RT-PCR, before normalizing them to a housekeeping gene. Consequently, the initial aims of the present study were to determine whether a linear response existed for detection of 28S, β-actin, β2-microglobulin (β2M), cyclophilin (CYC), and GAPDH gene expression in human skeletal muscle using raw data values and to characterize the real-time RT-PCR method in terms of intra- and interassay variability. We then aimed to identify genes, which in response to short-term CrS, were expressed with the least variation and to subsequently establish their use as a housekeeping gene in human skeletal muscle. The samples analyzed in this study were part of a larger study, and the protocol included a small number of high-intensity exercise bouts to investigate the potential ergogenic effects of Cr. Finally, to establish the power with which those housekeeping genes would be examined in the present study, RNA was obtained from individuals exposed to various interventions used in our laboratory (e.g., exercise; dietary intervention, training etc). This RNA was pooled and converted to cDNA using random hexamers. Resultant cDNA was stored at −80°C until subsequent analysis. All samples were being compared undergone RT together. For the PCR step, reaction volumes of 20 μl contained SYBR Green 1 Buffer (Applied Biosystems, Foster City, CA), forward and reverse primers (see Table 1), and cDNA template (see below) per tube. Samples were run in triplicate on one or two separate occasions (see below). Real-time PCR was run for 1 cycle (50°C 2 min, 95°C 10 min) followed immediately by 40 cycles (95°C 15 s, 60°C 60 s), and fluorescence was measured after each of the repetitive cycles. The fluorescence resulted from the incorporation of SYBR Green 1 dye into the double-stranded DNA produced during the PCR reaction, and emission data were quantitated using the threshold cycle (Ct) value. Ct readings obtained represent measurements on the log scale. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. To validate the specificity of a primer set, RNA (1–3 μg) and the RT negative were analyzed in triplicate to confirm that there was no fluorescence resulting from either genomic DNA contamination or from the RT step. Each PCR run also included triplicate wells of no template control (NTC) where RNase-free water was added to reaction wells.

Pilot study. A pilot study was conducted to determine which housekeeping genes would be examined in the present study. RNA was extracted from several samples of skeletal muscle obtained from individuals exposed to various interventions used in our laboratory (e.g., exercise; dietary intervention, training etc). This RNA was pooled and converted to cDNA using random hexamers. Resultant cDNA was added

### Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cellular Function</th>
<th>GenBank Accession Number</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Conc. μM</th>
<th>Reverse Primer (5’ → 3’)</th>
<th>Conc. μM</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>Ribosomal subunit, abundant</td>
<td>M11167</td>
<td>TTAGTGACGGCAGCATGAAATGG</td>
<td>2</td>
<td>TGTTGTTCCGCGATGATAGGT</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Highly conserved protein involved in cell motility</td>
<td>NM_001101</td>
<td>GACAGGATGGAGAAGGAGATTACT</td>
<td>2</td>
<td>TGATCCACATCTGCTGGAAGGT</td>
<td>2</td>
<td>141</td>
</tr>
<tr>
<td>β2M</td>
<td>Involved with immune response</td>
<td>XM_007650</td>
<td>ATGAGTATGGGCTGGGTGTA</td>
<td>2</td>
<td>GGCATCTCTCAAACCTCCATG</td>
<td>2</td>
<td>101</td>
</tr>
<tr>
<td>CYC</td>
<td>Cyclosporin A binding protein; involved in cellular protein, folding and interactions</td>
<td>XM_004890</td>
<td>CCAACCCTGTTCTCTGACAT</td>
<td>3</td>
<td>CCAGTGCCAGACAGAA</td>
<td>3</td>
<td>116</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Major glycolytic pathway enzyme</td>
<td>XM_006959</td>
<td>CGACCCCATGGGAAATGCC</td>
<td>3</td>
<td>TGAGATTCTGAGATCAGGAA</td>
<td>3</td>
<td>69</td>
</tr>
</tbody>
</table>

in duplicate to the commercially available Applied Biosystems Endogenous Control Plate (Applied Biosystems) and underwent PCR according to the manufacturer’s instructions. For each sample, a C_T value was obtained for each of the 12 genes present on the plate. The genes with the least variation in C_T values across all the interventions were found to be β-actin, β2M, and CYC. In addition to these genes, GAPDH was included, since it has been used in a previous study examining gene expression in human skeletal muscle following CrS (31). 28S was also examined, since it has been used as a representative ribosomal RNA previously (4, 8).

**Primers designs.** To perform PCR, specific primers were designed for the genes 28S, β-actin, β2M, CYC, and GAPDH, using Primer Express software (Applied Biosystems) on sequences attained from GenBank (see Table 1 for details). Searching of the sequences using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) confirmed their specificity. Primers were purchased from GeneWorks (Adelaide, SA, Australia).

**Relationship between starting cDNA amounts and PCR results.** To determine the validity of using raw C_T values as a measure of starting cDNA concentrations, real-time PCR was performed to measure the genes 28S, β-actin, β2M, CYC, and GAPDH. RNA extracted from a single human muscle sample was used to examine the dynamic range of response for a series of dilutions of cDNA generated from the RT step (see Table 2). Linear regression was used to analyze the response of C_T vs. the logarithm of the cDNA concentration. Using the slopes of the lines, we calculated the efficiency (E) of target amplification with the equation E = (10(-1/slope)) - 1 (Ref. 18).

**Linearity of RT step to PCR.** To ascertain whether, for the genes examined (28S, β-actin, β2M, CYC, and GAPDH), the RT step exhibited a linear response, RNA was extracted from the RT mix (optimal for RT of 1.0 μg RNA) to give 0.1 μg to 2.0 μg RNA, which subsequently underwent simultaneous RT followed by PCR. Linear regression was used to analyze the response of C_T vs. the logarithm of the amount of input RNA to the RT step. Efficiency was calculated as previously described.

**Intra-assay variability of RT-PCR.** To determine the intra-assay variability, the coefficient of variance (CV) was determined for samples run in triplicate for detection of β-actin, β2M, CYC, and GAPDH. C_T values were calculated using the term 100 × (standard deviation/mean). A total of 94 triplicate readings were used over the 4 genes. The CV was determined for the raw C_T values, and also for the term 2^(-C_T), which converts the log scale C_T value to the linear form.

**Interassay variability of RT-PCR.** To determine interassay variability, the CV was calculated for triplicate readings of samples (n = 34), which were run over two separate occasions (Table 3). For the four genes examined, the two occasions were separated by up to 39 days (21 ± 3 days, mean ± SE, see Table 3). The CV was also calculated for the 2^(-ΔC_T) values.

**Biological and technical variability.** Technical variability incorporated inconsistencies due to dilutions, as well as instrumentation variation in OD260 readings and C_T values obtained from the PCR. To determine the variability due to the dilutions, six aliquots of total RNA extracted from a single muscle sample were quantified on a spectrophotometer measuring the OD260. The variability in the OD260 readings was measured in the same samples (n = 3) over three occasions. To evaluate the technical variability due to the RT step, six aliquots of a single RNA sample underwent RT (0.5 μg total RNA per 10 μl reaction) followed by PCR in triplicate. To determine the error associated with biological variability, a single skeletal muscle sample was cut into seven pieces (7.1 ± 1.4 mg, mean ± SD), and total RNA was extracted simultaneously from each piece. Subsequent to quantification of the total RNA from all samples, 0.5 μg RNA underwent RT in 10 μl. Following RT, the cDNA from all samples was diluted and underwent PCR in triplicate using β-actin primers. To determine the combined technical and biological variability of the samples, the variation in the C_T value obtained for the given samples was calculated. To establish the contribution due to biological variability, the average of the technical variability due to the RT and PCR steps involved in obtaining a C_T value was calculated and subtracted from the overall variability.

**Study design for the determination of an appropriate housekeeping gene when skeletal muscle Cr content was elevated by oral Cr supplementation.** Healthy, active male subjects, aged 25.4 ± 4.6 yr (mean ± SD) were used in the study, which was approved by Deakin University Human Ethics Committee and was part of a larger study being conducted by us. The study group consisted of habitual meat eaters (n = 4) and non-meat eaters (n = 4). Subjects (n = 8) underwent a double-blind, crossover study involving the ingestion of Cr (Cr, 0.4 g/kg body wt^(-1)-day^(-1) plus glucose, 0.4 g/kg body wt^(-1)-day^(-1); CR group) or a placebo (glucose, 0.8 g/kg body wt^(-1)-day^(-1); placebo group). Treatment order was randomly assigned and was separated by 5 wk. The washout period of intramuscular Cr has previously been shown to be 4 wk (9, 20). Resting muscle biopsies were taken from the vastus lateralis as previously described (26) before the supplementation period (day 0) and following 1 day (day 1) and 5 days (day 5) of supplementation using the percutaneous needle biopsy method modified to include suction. The muscle samples were stored in liquid nitrogen until analyzed. Immediately after each biopsy, two 30-s all-out sprints, separated by 4 min of passive rest, were performed on a cycle ergometer (Excalibur ergometer; Lode, Groningen, The Netherlands). Consequently, the data correspond to the effects of the ingestion of Cr compared with a placebo when both groups perform this exercise.

**Total Cr content.** To ensure that all subjects responded to CrS, intramuscular total Cr (TCr) measurements were made. Muscle samples were freeze-dried for 24 h, weighed and powdered, removing any visible connective tissue, and then extracted using 0.5 M perchloric acid and 1 mM EDTA, and neutralized with 2.1 M KHCO_3. These extracts were subsequently analyzed for creatine phosphate (CrP) and Cr levels using an enzymatic fluorometric technique (19). TCr was taken as the sum of CrP and Cr.

**Examination of housekeeping genes.** Based upon the results of the validity and reliability studies, the protocols for
which are outlined above, four genes were chosen to establish their consistency of expression in human skeletal muscle biopsies at 0, 1, and 5 days postsupplementation with Cr or placebo. To reduce intersubject variation, samples were normalized to the day 0 sample of the placebo trial for each subject. For \( \beta \)-actin, 2M, CYC, and GAPDH, a \( \Delta C_T \) value was calculated for each sample by subtracting the \( C_T \) value for a given sample from the \( C_T \) value of the gene being treated as the housekeeping gene (except when this was the same gene). The relative expressions of these genes compared with the basal values were calculated using the expression \( 2^{-\Delta\Delta C_T} \). The efficiency of the RT-PCR for \( \beta \)-actin, 2M, and CYC was determined to be the same across a series of dilutions (data not shown), thus justifying their use as housekeeping genes.

### Power analyses

To reveal the differences measurable in the current experiments, power analyses were performed for each gene using the equation

\[
n = 2S^2/\Delta^2 \times f(\alpha, \beta)
\]

where \( n \) = required sample size per group; \( S \) = estimate of standard deviation (average of triplicate readings from \( n = 24 \) for each gene); \( \Delta \) = desired width of confidence interval (CI) in \( C_T \); \( (1 - \alpha) = 95\% \) CI; \( (1 - \beta) = \) power; \( f(\alpha, \beta) \) was adopted for a two-tailed test.

**Table 3. Coefficient of variance for triplicate readings for intra-assay (column A) and interassay (column B) assay variability using raw \( C_T \) values or \( 2^{-\Delta C_T} \) values**

<table>
<thead>
<tr>
<th></th>
<th>A: CV from Triplicate readings, Same Samples in the Same Run</th>
<th>B: CV from Triplicate Readings Across Separate PCR runs</th>
<th>Samples Measured in Triplet</th>
<th>Samples Measured in Triplet Over 2 Runs</th>
<th>Days Separating 2 Runs (means ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta )-Actin</td>
<td>1.4% 25%</td>
<td>1.4% 35%</td>
<td>6</td>
<td>10 ± 9</td>
<td></td>
</tr>
<tr>
<td>2M</td>
<td>1.4% 20%</td>
<td>2.4% 34%</td>
<td>9</td>
<td>31 ± 13</td>
<td></td>
</tr>
<tr>
<td>CYC</td>
<td>1.1% 20%</td>
<td>1.5% 26%</td>
<td>8</td>
<td>31 ± 17</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.6% 21%</td>
<td>3.3% 39%</td>
<td>11</td>
<td>8 ± 8</td>
<td></td>
</tr>
<tr>
<td><strong>Overall CV</strong></td>
<td><strong>Total 1.3% 21%</strong></td>
<td><strong>Total 2.3% 34%</strong></td>
<td><strong>34</strong></td>
<td><strong>21 ± 16</strong></td>
<td></td>
</tr>
</tbody>
</table>

CV, coefficient of variance; \( C_T \), cycle threshold.

### Statistics

BMDP software was used to perform two-way (treatment \( \times \) time) ANOVAs. TC\( r \) results are expressed as means ± SE; 95\% CI values, slopes, and y-intercepts were calculated using Prism GraphPad Software.

**RESULTS**

Amplification of all genes was as expected, with a region of exponential increase in fluorescence preceding a plateau phase. Following RT-PCR, the samples were subjected to a heat dissociation protocol over 60–95°C, indicating the number of products present in each reaction well, and the temperature at which the products dissociated (Fig. 1). RNA, RT negative, and NTC produced no amplification (i.e., remained below threshold) during the 40 cycles of PCR, confirmed by neither a \( C_T \) value nor a dissociation curve being obtained (data not shown). This substantiated the absence of genomic DNA or primer dimer formation, which could otherwise contribute to observed fluorescence. Threshold values were set to cross the exponential phase of each gene’s amplification, and from this the \( C_T \) was determined. The amount of transcript present is inversely related to the observed \( C_T \), and for

**Fig. 1. Real-time RT-PCR analysis of skeletal muscle mRNA content. Typical heat dissociation protocol for cyclophilin (CYC). Following PCR, the samples and no template control (NTC) were subjected to a heat dissociation protocol over 60–95°C. Samples yielded a product, which dissociated at 83°C, whereas the NTC had no product present.**
every twofold dilution in transcript a single increase in
CT is expected to be observed.

**Linearity of various input cDNA amounts to PCR.** For each gene, the validity of using C_T values was established, since a linear relationship existed between the C_T values and the logarithm of the cDNA template concentration (Fig. 2). Dilutions of cDNA exhibited five orders of dynamic range for C_T values for 28S, β-actin, β2M, CYC, and GAPDH ($r = -0.9786$, $-0.9581$, $-0.9255$, $-0.9465$, and $-0.9066$, respectively; Fig. 2). The mean efficiency ($E$) of the PCR reaction for 28S, β-actin, β2M, and GAPDH was 1.1 and for CYC was 0.9 (see table in Fig. 2). A 100% efficient PCR would produce a slope of $-3.3 (E = 1)$, indicating that twice as many amplicons are being made for every PCR cycle, represented as a decrease of 1 C_T. In addition, the 95% CI values for the slope values of the graphs (see table in Fig. 2) indicate a linear detection of the cDNA for all genes.

**Linearity of RT step to PCR.** Gene expression of 28S exhibited a positive relationship ($r = 0.5180$) between input RNA amounts and C_T values obtained (data not shown). As the amount of input RNA increased, the C_T value increased which indicates a decrease in input template, where there was actually an increase in template. Consequently, 28S was excluded from subsequent experiments testing for a valid housekeeping gene. C_T values were not correlated to the logarithm of the input RNA when either too little ($\sim 0.10 \mu g$) or too much ($\sim 2.0 \mu g$) RNA was added to the RT reaction (data not shown). When the upper and lower concentrations were omitted as outliers, β-actin, β2M, CYC, and GAPDH exhibited inverse correlations ($r = -0.9230$, $-0.8787$, $-0.8130$, and $-0.7336$, respectively) between

![Fig. 2](image_url)
CT values and the logarithm of the input RNA content (Fig. 3). The efficiencies of the PCR reactions were 1.4, 1.1, 1.3, and 1.2 for β-actin, β2M, CYC, and GAPDH, respectively (see table in Fig. 3). Examination of the 95% CI values for the slopes of each gene indicated that the efficiencies were not different.

Intra- and interassay variability of RT-PCR. Calculation of the CV for CT values and the conversion of the CT values to their linear form using the expression $2^{-\Delta \text{CT}}$ was determined for triplicate readings for βH252–actin, βH2522M, CYC, and GAPDH for intra-assay (Table 3, column A) and interassay (Table 3, column B) variabilities, respectively. Overall, the intra-assay variability was 1.3% and 21% and interassay variability was 2.3% and 34% for raw CT values and $2^{-\Delta \text{CT}}$, respectively.

Biological and technical variability. The technical variation due to the OD260 readings obtained for a series of six aliquots of the same total RNA extract was $0.050 \pm 0.003$ (mean ± SD; CV, 6.2%; n = 6). This value incorporates a 5.0% variation due to the repeatability of OD260 readings. Once the validity of using raw CT values for β-actin was determined, the biological variation seen in different extractions from the same piece of muscle was examined. Variability in the raw CT values for replicates of a single sample due to the RT step was $24.0 \pm 0.6$ (mean ± SD; CV, 2.6%; n = 7; Table 4). Across the samples, variability in the raw CT values obtained was $24.1 \pm 1.5$ (mean ± SD; CV, 6.2%; n = 7; Table 4), which accounts for both technical (incorporating inconsistencies in OD260 readings) and biological variability. To determine the biological variability, the variation of the two technical steps (RT 2.6% and PCR 1.9%) involved in obtaining a CT value were averaged, then subtracted from the overall variability. Using this procedure, we found that the biological variability accounted for 4.3% of the overall variability (Table 4).

Intramuscular total Cr content. Compared with both CR and placebo day 0 concentrations, intramuscular TCr levels were increased in the CR trial ($P < 0.05$) at day 1 (+22 ± 4%) and day 5 (+54 ± 9%; Fig. 4). In the CR trial, day 5 intramuscular TCr was also higher ($P < 0.05$) than day 1 (+26 ± 6%). Although there were no differences between any of the time points in the placebo trial, the CR day 0 was lower than day 0 in the placebo trial, probably due to incomplete washout of intramuscular TCr stores in the four subjects who received Cr in the first trial. A 4-wk washout period

### Table 3: Regression equations and efficiency values

<table>
<thead>
<tr>
<th>Gene</th>
<th>$r^2$ value</th>
<th>Regression equation</th>
<th>95% confidence intervals, slope</th>
<th>Mean efficiency $(10^{-\text{slope}} - 1)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclophilin</td>
<td>0.6610</td>
<td>$y = -2.70 x + 28.5$</td>
<td>-3.41 to -1.99</td>
<td>1.3</td>
</tr>
<tr>
<td>β-actin</td>
<td>0.8519</td>
<td>$y = -2.69 x + 25.9$</td>
<td>-3.08 to -2.30</td>
<td>1.4</td>
</tr>
<tr>
<td>β-2-microglobulin</td>
<td>0.7721</td>
<td>$y = -3.09 x + 21.47$</td>
<td>-3.69 to -2.50</td>
<td>1.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.5382</td>
<td>$y = -2.88 x + 17.41$</td>
<td>-3.83 to -1.93</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Fig. 3.** Dose response curves following RT-PCR for 0.25 to 1.5 μg total RNA (plotted as log values) added to the RT reaction vs. CT. Values are the means of 3–6 readings over 2 PCR runs for the genes β-actin (•), β2M (○), CYC (●), and GAPDH (■). Values are means with 95% CI values indicated by dashed lines. **Bottom:** linear regression equations, 95% CI values for slopes, and efficiencies ($E = 10^{-1/\text{slope}} - 1$) are indicated for each gene.

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has previously been shown to result in intramuscular TCr stores returning to pre-CrS values (9, 26). TCr values at days 1 and 5 in the CR trial were greater than all time points in the placebo trial (Fig. 4).

**Examination of housekeeping genes using raw CT values.** Having determined the linear nature of CT values vs. the logarithm of cDNA and input RNA amounts, with no difference in the efficiencies between the genes, CT values were subsequently used as a measure of specific mRNA contents in samples. RNA was extracted from human skeletal muscle samples obtained at days 0, 1, and 5 from the CR and placebo trials and underwent RT-PCR in triplicate for β-actin, β2M, CYC, and GAPDH. mRNA expression was expressed as the fold change in CT values relative to the CT value at day 0 in the placebo trial (Fig. 5). There was a tendency for a main effect for time for GAPDH (P = 0.08). The 95% CI values have been plotted, which indicate that GAPDH was the most variable gene following CrS, while β-actin, β2M, and CYC showed similar variations of within ~1 CT value.

**Examination of housekeeping genes using normalized CT values.** To determine a normalized arbitrary value (2^ΔΔCT) for each gene, each data point was normalized to the least varying genes (i.e., β-actin, β2M, and CYC), as well as to the placebo day 0 CT value. CYC normalized to β-actin (Fig. 6A) and β-actin normalized to CYC (Fig. 6D) were not different between the trials. When normalized to β-actin, β2M gene expression was different (P < 0.05) between placebo day 0 and day 5 samples (Fig. 6B). There was significant interaction (P < 0.05) for GAPDH (Fig. 6C) when normalized to β-actin. When normalized to CYC, there were tendencies for main effects for time for β2M (P = 0.06; Fig. 6E) and GAPDH (P = 0.08; Fig. 6F). When normalized to β2M, there were no differences in the expression of β-actin (Fig. 6G), CYC (Fig. 6H), or GAPDH (Fig. 6I).

**Power analysis.** The power with which a 95% CI could be obtained using the data set in the present study (i.e., n = 8) are listed in Table 5. The conventional power = 0.8 indicated that for a group of eight subjects a difference of 1.0–1.2 CT is measurable in all genes except 28S, for which a 3.1 CT difference could be detected.

**DISCUSSION**

The present study examined the validity and reliability of measuring the expression of various genes in human skeletal muscle using real-time RT-PCR. We examined the relationships between starting RNA concentrations and input cDNA amounts against the CT values obtained for 28S, β-actin, β2M, CYC, and GAPDH, as well as the intra- and interassay CV values. For data analysis, an endogenously expressed gene, or a gene that does not vary significantly across the given intervention, known as a housekeeping gene, is required for normalization of the genes of interest. As such, the technique assumes prior knowledge about the least changing behavior of the housekeeping gene with respect to the specified intervention. In the current study, the validity of using certain genes as housekeeping genes when human skeletal muscle was exposed to elevated total creatine levels was examined.

**Linearity of various input cDNA amounts to PCR.** The first experiment investigated the dynamic range of quantitation of RT-PCR using Ct values. Following the log conversion of a series of fold dilutions from the same cDNA, a linear response was demonstrated for all genes tested (28S, β-actin, β2M, CYC, and GAPDH; Fig. 2). Examination of the 95% CI showed that for all genes the slope of the logarithmic relationship included ~3, which indicated that the PCR was able to detect twofold differences in input template, reflected by a single CT value. Hence, these data indicated that the amounts of cDNA added to a given PCR run were reflected by differences in CT values for 28S, β-actin, β2M, CYC, and GAPDH transcripts. This finding validates the use of the CT values for detection of absolute changes in the amount of input cDNA for these particular transcripts. These data are in agreement with other researchers who have reported the efficiency of real-time RT-PCR using SYBR Green 1 chemistry and various genes in different tissues (6, 21, 24).

**Linearity of RT step to PCR.** Linear relationships between the logarithm of various input amounts of RNA into the RT reactions and the CT values of β-actin, β2M, CYC, and GAPDH were obtained (Fig. 3), once the lowest and highest input RNA amounts were omitted. The addition of either not enough (e.g., ~0.1 μg RNA) or too much (e.g., ~2.0 μg RNA) RNA into an RT reaction, optimal for the RT of 1.0 μg RNA, appeared to be inhibitory to the reaction (data not shown). Although the reactions were not 100% efficient for all the genes, the important feature is that since the 95% CI for the slopes of the graphs for each gene overlapped

### Table 4. Biological and technical variability in measuring mRNA expression using real-time RT-PCR

<table>
<thead>
<tr>
<th>Parameter Being Measured for Variability</th>
<th>Sample Size (in triplicate)</th>
<th>CT Values (means ± SD)</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT (technical)</td>
<td>7</td>
<td>24.0 ± 0.6</td>
<td>2.6%</td>
</tr>
<tr>
<td>RT-PCR (from Table 3, technical)</td>
<td>16</td>
<td>24.9 ± 1.0</td>
<td>1.4%</td>
</tr>
<tr>
<td><strong>Average technical variability 1.95%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR (biological and technical)</td>
<td>7</td>
<td>24.1 ± 1.5</td>
<td>6.2%</td>
</tr>
<tr>
<td><strong>Overall biological variability = Biological and technical less technical variability 4.25%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
each other (see table in Fig. 3), the efficiency of the AMV reverse transcriptase enzyme was not different between β-actin, β2M, CYC, and GAPDH. These data suggest that for samples undergoing RT at the same time, RT efficiency appears to be the same for input RNA amounts between 0.5 to 1.5 \( \mu \)g and that the subsequent analyses for determination of an appropriate housekeeping gene(s) were valid. The response for 28S was different from the other genes examined, since it exhibited a positive relationship between input RNA amounts of 0.5 to 1.5 \( \mu \)g vs. \( C_T \) values (data not shown). It is not known why 28S responded in this way, although we speculated that the use of the oligo dT primers, which would selectively reverse transcribe RNA species possessing a poly-A tail, would not be suitable for efficient translation of ribosomal RNA (i.e., 28S). To test that possibility, we undertook RT reactions using random hexamers (Applied Biosystems) using various input amounts (0.25 to 1.5 \( \mu \)g RNA) of pooled RNA. In our hands, this also failed to display an inverse linear relationship (Fig. 7), and as such we eliminated the further testing of 28S in the current experiments. We do not know why this was the case; however, it is possible that the relatively high abundance of the ribosomal RNA may somehow impede the detection of the transcripts in human skeletal muscle using SYBR Green 1 chemistry and the sensitive real-time RT-PCR methods. Consequently, the choice of 28S is not appropriate for real-time PCR under the conditions employed by us (i.e., SYBR Green 1 chemistry, oligo dT or random hexamers as cDNA priming oligo, and two-step RT-PCR). It is possible that 28S might be an appropriate housekeeping gene for human skeletal muscle under other specific conditions (e.g., use of gene-specific primers as cDNA priming oligo, use of TaqMan probe with one-step RT-PCR, or greater dilutions of RNA), although these possibilities were not examined in the current study. To our knowledge, the examination of the efficiency of the RT enzyme AMV on high- or low-abundance target genes has not been reported and might warrant further investigation. Interestingly, it has been demonstrated that the efficiency of the RT enzyme, Moloney murine leukemia virus (MMLV), fell from \( \approx 20\% \) to less than 6\% when a low-abundance target template was used (7). Furthermore, it has recently been reported that the choice of cDNA synthesis conditions influences the sensitivity and accuracy of real-time PCR using SYBR Green 1 chemistry (18). Those authors also found that the efficiency is related to both choice of RT primer as well as transcript being investigated (18). As mentioned in the previous section, the use of fivefold dilutions of cDNA and measurement of \( C_T \) values demonstrated the linearity of the system for determining the input amount of serial dilutions of 28S rRNA. The data obtained using different amounts of RNA input in the RT reaction along with the findings of Lekanne Deprez and colleagues (18) further highlight the importance, emphasized by numerous researchers previously (11, 14,
that investigators need to optimize parameters for individual experimental conditions. To our knowledge, this is the first study that has examined the linearity of the RT step to PCR using RNA obtained from skeletal muscle.

Intra- and interassay variability of RT-PCR. CV values for triplicate CT readings were 1.3 to 2.3% for intra- and interassay variability, respectively. These values are in line with those reported by others (5, 23). Interestingly, Schmittgen and colleagues (23) pointed out that presentation of statistical data calculated using these raw CT values was incorrect due to the logarithmic nature of CT values. Using this approach, we recalculated the CV using the transformation of CT to the linear $2^{-\Delta C_T}$ value, where the variability was seen as 21 to 34% for intra- and interassays. These values are

![Fig. 6: Gene expression at days 0, 1, and 5 from creatine (CR; solid symbols) and placebo (open symbols) trials (n = 8). Data are expressed as $2^{-\Delta C_T}$ values obtained by normalizing CT values to day 0 of the placebo trial and to $\beta$-actin (A, CYC; B, $\beta_2$M; and C, GAPDH), CYC (D, $\beta$-actin; E, $\beta_2$M; and F, GAPDH), or $\beta_2$M (G, $\beta$-actin; H, CYC; and I, GAPDH). Values are means with 95% confidence are indicated by dashed lines. *P $<$ 0.05 different to day 0.](fig6.png)

![Fig. 7: Dose response curve following RT-PCR for 0.25 to 1.5 μg total RNA added to the RT reaction vs. CT for 28S. cDNA underwent RT using random hexamers primers. Values are means with 95% CI values indicated by dashed lines.](fig7.png)

Table 5. Power analyses for the determination of detectable limits using real-time RT-PCR

<table>
<thead>
<tr>
<th>Power</th>
<th>0.9</th>
<th>0.8</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-Actin</td>
<td>3.84</td>
<td>7.85</td>
<td>10.51</td>
</tr>
<tr>
<td>$\beta_2$M</td>
<td>1.3</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>CYC</td>
<td>1.2</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.4</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>28S</td>
<td>3.6</td>
<td>3.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

CI, confidence interval.
similar to that reported for intra assay CV using $2^{-\Delta \Delta CT}$ (23).

**Biological and technical variability.** The same piece of muscle cut into seven pieces resulted in a CV of 6.2%, which included both biological (within the same sample, 4.3%) and technical error (1.9%; see Table 4). It needs to be emphasized that this total variability is likely to be the minimum, since further intra-individual variation is likely when additional muscle samples from the same subject are analyzed, due variation in parameters such as fiber type (2). Nonetheless, these data highlight the areas of variability present within a single skeletal muscle sample when real-time RT-PCR is the tool of measuring mRNA expression. Although previous researchers have attributed large variability in uncoupling protein (UCP) 2 and UCP3 gene expression in muscle samples obtained from the same subject to biological variability, no quantitative data were provided for variation within a sample (3), and to our knowledge the current paper is the first to provide these data. Additionally, the current study did not measure intersubject variability, although CV values of 50–70% have been reported for the expression of UCP2 and UCP3 gene expression in skeletal muscle amongst individuals (3).

**Cr supplementation and gene expression.** Only a single study has examined gene expression following Cr supplementation (31). In that study it was reported that skeletal muscle myosin heavy chain (MHC) I, IIa, and IIX gene expression was higher following 12 wk of resistance training combined with Cr supplementation compared with resistance training and a placebo (31). This increase in MHC gene expression was attributed to a synergistic response between the resistance training and Cr supplementation. In terms of proteins, the ingestion of Cr resulted in differential protein expression of the myogenic transcription factors myogenin and MRF4 following a period of immobilization and 10 wk of resistance training (15). Data from these studies indicate that the examination of gene expression consequent to Cr supplementation warrants further investigation. It is important to note that outcomes of studies examining the effect of Cr supplementation on gene expression are dependent on the knowledge of an appropriate housekeeping gene.

**Examination of housekeeping genes using raw CT values.** Following the characterization of real-time RT-PCR, we examined the response of $\beta$-actin, $\beta_2$M, CYC, and GAPDH mRNA at days 0, 1, and 5 following ingestion of either Cr or placebo interspersed with high-intensity exercise. The Cr trial showed increased intramuscular TCr stores at days 1 and 5 compared with day 0, as well compared with the placebo trial, indicating a response to the supplementation period. Initial examination of the effect of Cr supplementation on gene expression by two-way ANOVA showed no statistical differences for any of the genes (Fig. 5), although there was a trend ($P = 0.08$) for a main effect for time for GAPDH. Inspection of the 95% CI indicated that GAPDH was the most variable gene, with the remaining genes behaving in a similar manner. The variability in the gene expression of GAPDH would indicate that this is not a good choice of housekeeping gene and that its use may mask differences that are in fact present. This would suggest that $\beta_2$M, $\beta$-actin, or CYC could be appropriate housekeeping genes for the present study.

**Examination of housekeeping genes using normalized CT values.** Since initial examinations suggested that only GAPDH showed variable expression, $\beta_2$M, $\beta$-actin, and CYC were used for the next step of normalizing the $2^{-\Delta \Delta CT}$ data to $2^{-\Delta \Delta CT}$ values. Normalization of the $\beta$-actin and CYC data to each other showed a similar stability in their expression. Interestingly, when the $\beta$-actin and CYC were normalized to $\beta_2$M, their expression did not change (Figs. 6G and 6H). Additionally, the expression of $\beta_2$M normalized to CYC was not different (Fig. 6E), although the expression of $\beta_2$M was different between days 0 and 5 in the placebo trial when normalized to $\beta$-actin (Fig. 6B). The inconsistent expression of $\beta_2$M suggests that it would not be an appropriate choice of housekeeping gene compared with $\beta$-actin and CYC. This discrepancy also highlights the importance of the correct choice of housekeeping gene, particularly when only small differences in the order of twofold changes may be present. It could in fact support the suggestion of employing two housekeeping genes (28), as well as using various baselines for normalizing data, as demonstrated by Hortobagyi and colleagues to validate their data (16). The change in $\beta_2$M, a gene involved in immune response (1), in the placebo trial at day 5, but not the CR trial, could suggest that this gene is responding to the exercise undertaken during the trial and that this effect is attenuated by the Cr supplementation. Examination of this possibility is outside the scope of this paper; however, further investigation is warranted. There were tendencies for main effects for time for GAPDH when normalized to either $\beta_2$M or CYC. A significant interaction with no changes detected by post hoc analysis was seen when GAPDH was normalized to $\beta$-actin. Examination of the GAPDH data indicated it was the most variable gene measured in this study. The variable nature of this gene across various interventions and between individuals is highlighted in a recent review (5). As such, its use as a housekeeping gene has been questioned previously, and it would seem that Cr supplementation in combination with high-intensity exercise also introduces wide variation in the expression of GAPDH and it would not be recommended as a housekeeping gene for this intervention. Interestingly, the only other studies that have examined the effect of Cr on skeletal muscle gene expression have adopted GAPDH as their endogenous control (31). Justification of using GAPDH due to its constitutive nature was based on cultured cell data (31). Our results point to the possibility that the results may have been affected by large variations in the choice of housekeeping gene in these studies.

**Power analyses.** RT-PCR is typically associated with large variability (5), although far less than other methods of mRNA detection. A previous report examining
housekeeping genes suggested that for most genes, less than fourfold differences are probably not biologically significant (30); however, it should be added that we currently are unable to say what magnitude of fold difference in gene expression would be required to represent a biologically significant outcome. Our data indicate that, with real-time RT-PCR, approximately a twofold or greater difference in the gene expression of β-actin, β2M, CYC, or GAPDH is measurable with 0.8 power and 95% confidence, for n = 8. For 28S a six- to eightfold difference would be required for detection at this power level and sample size.

Conclusion. We have demonstrated the quantitative and linear detection of amplified products from human skeletal muscle using SYBR Green 1 chemistry and real-time RT-PCR and the variability of triplicate readings using this method. When taking into account all experiments of the present study, it would appear that β-actin and CYC would be the most valid choices of housekeeping genes for real-time RT-PCR analysis of human skeletal muscle samples exposed to short-term CrS and high-intensity exercise.

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