Efficient experimental design and analysis of real-time PCR assays

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Introduction

Real-time PCR (qPCR) has become the definitive approach to quantify gene expression and corroborate high-throughput microarray results in both basic sciences and clinical research. Because of the sensitivity of qPCR to subtle experimental variations, a list of guidelines, known as Minimum Information for publication of quantitative real-time experiments (MIQE),1 was recently established to ensure the integrity, consistency, transparency and reproducibility of qPCR results. Because of the comprehensive nature of the guidelines, adherence can be impractical and costly (both financial and labor) for some.

The traditional qPCR design strategy2 comprises the determination of (1) the PCR efficiency for each primer pair and (2) the target DNA quantity for all samples, with technical variations assessed by performing identical replicates for all reactions. As the number of primer pairs and samples increase, the number of reactions can become overwhelming. Moreover, the design assumes that the PCR efficiency is invariant across all samples, preparations and reactions. However, there has been no assessment of whether this experimental design reflects the minimal sufficient number of reactions required to provide a statistical determination of gene expression levels.

In this paper, we described an experimental design based on the traditional qPCR approach by reducing fewer reactions of DNA samples. The design takes into account the notion that each qPCR reaction yields a Cq value reflecting both initial target gene quantity and reaction efficiency. We verify this novel design on a well-characterized model and develop a simple analysis procedure capable of robustly quantifying gene expression levels even against large variations in individual reactions. The proposed experimental design and analysis strategy streamlines qPCR experimentation, providing more affordable and time-efficient means to scale up gene expression studies.

Results

Description of myocardial hypertrophy model. Phenylephrine (PE) treatment leads to enlargement of cells,3 and has been used as a well-studied model in studying cellular hypertrophy.4 Atrial natriuretic factor (ANF) is a biomarker (upregulated gene expression) for PE-induced hypertrophy in myocytes.5 PE treatment results in the phosphorylation, but not altered gene expression, of extracellular signal-regulated kinase-2 (ERK2, also known as mitogen-activated protein kinase 1, MAPK1; GenBank no. NM_053842).6 Consequently, an examination of ANF (GenBank no. M27498) and ERK2/MAPK1gene expression in PE-treated myocytes is useful in the evaluation of our novel qPCR experimental design strategy. For comparison, we selected two commonly used reference genes in hypertrophy and cardiopathological models, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank no. NM_017008)7,8 and the 40S ribosomal protein S16 (Rps16; GenBank no. NM_001169146).9,10

Design of experiment: simultaneous measurement of efficiency and quantity. The progression of a PCR (amplification) reaction with efficiency E, follows a standard exponential function,

\[ Q(n) = Q(0) \times E^n \]

Where Q is the quantity of product, n is the cycle number and Q(0) represents the initial quantity. For a defined threshold, T, in the rising phase of the amplification reaction, Cq is defined as the estimated cycle number at which Q crosses T. This is the customary value measured in qPCR experiments to estimate of the initial template quantity.

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Equation 3 indicates that the semi-log plot Cq vs. \( \log(d) \) has a slope of \(-1/\log(E)\), from which E can be evaluated and used to estimate initial quantities, \( Q(0) \), from test samples via Equation 1 (Fig. 1A). This traditional approach is known as the efficiency correction method of qPCR. Further note that, assuming E is indiscriminately constant, the y-intercept of this plot, \( \log(T/Q(0))/\log(E) \), is an indirect estimate of the initial

Figure 1. Proposed dilution-replicate experimental design and analysis. (A) The standard curve plot. Derived from the qPCR run of a single sample at multiple dilutions, the efficiency of the reaction is estimated from the slope of the linear fit to the semi-log plot. In the traditional qPCR experimental design, the efficiency is estimated from the average of 2–3 independent samples. (B) Cq-Cq plot. The analytic approach we propose to estimate relative gene expressions from the dilution-replicate experimental design. The expression ratio of a gene of interest (g) and a reference gene (r) is proportional to the intercept, as described in Equation 5. (C) Comparison of traditional and dilution-replicate qPCR experimental designs. A portion of the plates are shown, with three primer pairs, three test samples, and a single standard curve (for C1 only). (C1) In the traditional qPCR design, standard curves are generated from five dilution points for each primer pair with identical triplicates performed for each reaction. Only one of the two to three standard curves are shown here, and two of the three replicates are shown for primer pair 3. (C2) In our proposed dilution-replicate design, each test sample is serially diluted to three levels, which is used to construct both 3-point standard curves and Cq-Cq plots. Since each sample provides an estimate of the reaction efficiency, the result is three independent standard curves. Moreover, because there is no need for an independent sample standard curve, much fewer reactions are required for the experiment (compare number of filled wells in traditional vs. dilution-replicate designs).
quantity. The value of T can be determined from a reaction using a sample of known initial quantity. Alternatively, the relative difference in quantities between two samples can be ascertained by noting that this y-intercept value is more negative with larger Q(0). Consequently, the semi-log plot Cq vs. log(d), also known as the standard curve, simultaneously estimates both the PCR efficiency and the initial quantity.

To take advantage of the semi-log plot described by Equation 3, we created alternative experimental design based on the traditional method. In the traditional design, each reaction is identically replicated (usually three times) for each sample, resulting in an average Cq value used to estimate the initial quantity (Fig. 1C1). The PCR efficiency is separately evaluated using standard curves estimated from 2–3 independent samples. Additionally, if multiple runs are required, MIQE guidelines recommend that at least one identical (replicated) sample be present during each run to control for inter-run variation. Our proposed experimental design uses dilution-replicates instead of identical replicates. Specifically, a single reaction is performed on several dilutions for every test sample, similar to the design of a standard curve but without identical replicates at each dilution (Fig. 1C2). Since each dilution-replicate sample estimates the PCR efficiency, there is neither a need to estimate it independently nor a need for a common sample to evaluate inter-run variation (every sample estimates E, which can be used to evaluate inter-run variation). Consequently, the dilution-replicate experimental design can result in fewer reactions than the traditional design strategy of qPCR.

To illustrate the benefits of the proposed experimental design, dilution-replicate sample standard curves were created for several genes from the hypertrophic myocytes (Fig. 2A). Three levels of dilution were used: two of the samples (up and downward triangles) were diluted 2-, 10- and 50-fold, and the other two samples (squares and circles) were diluted 5-, 50-, and 500-fold. This spread in dilutions allowed us to examine how low samples (squares and circles) were diluted 5-, 50-, and 500-fold, and the other two (triangles) were diluted 2-, 10- and 50-fold, and the other two (solid circles) in the standard curve plots for the reference (Rps16 and GAPDH) and the PE-invariant (ERK2/MAPK1) genes overlap, indicating that the initial quantities of these genes in all the samples were similar. In contrast, for the ANF plots, all the PE-treated samples were consistently downward shifted (more negative y-intercept) compared with their controls, reflecting the fact that PE enhances ANF gene expression, again confirming Equation 3. A single control sample (solid circle) exhibited consistently higher Cq values for all genes compared with all other samples, which was the result of myocyte loss immediately prior to the isolation of total RNA. We opted to keep this control sample to assess its impact on the gene expression analysis. The myocyte hypertrophy example shows that the dilution-replicate design, in conjunction with the standard curve and Equation 3, can provide sufficient qualitative information to compare the expression levels of a gene from different samples while minimizing the necessary number of qPCR reactions. However, to quantify this difference in expression level, we still require an estimate of the qPCR efficiency, E.

Estimate of global PCR efficiency by collinear fit of standard curves. The accurate estimate of E, as measured by the reproducibility of the standard curve, is crucial to the accurate quantitative interpretation of qPCR experiments. Ideally, Cq values lie between 20 and 30 cycles and E approximately 2, with 2 representing 100% efficiency in replication. In addition to the variations in Cq, which arise from technical or instrumental error, there are variations in E, which have exponential effects on the estimate of the initial quantity. For example, a 0.5 variation in Cq results in a 2−0.5 - 20.5 or 29–41% misestimate if E = 2, whereas a 0.05 error in E results in a (1.95/2)30–(2.05/2)30 or 53–110% misestimate after 30 cycles if E = 2. Consequently, it is important to determine E as accurately as possible for the set of primers and samples.

In the traditional qPCR experimental design, the value of E is determined by averaging results from standard curves of 2–3 independent samples from 4–5 dilution points. Although estimates of E can also be independently performed for every sample in the dilution-replicate design, there is an alternative approach which benefits from the unique design strategy. Assuming that E is constant across all samples, all standard curves can be simultaneously fit with the constraint of slope equality, resulting in a globally estimated E. There are three points to note for this approach. First, there are [(dilution points −1) × (number of samples) −1] degrees of freedom in this fit, indicating that less dilution points are needed with more samples (though a minimum of three is recommended to sufficiently describe the linear fit and its variability for each sample). Second, when the dilutions are identical between all samples, the average of independently fit slopes yields the same result as a least squares constrained globally fit slope. However, when the dilutions are different between samples, the constrained fit slope is less affected by large errors in individual Cq values than the average from independent fits. Third, since the constraint forces a global slope on all samples, it effectively acts like an extra point in each sample standard curve. Consequently, when this constrained fit is performed on the dilution-replicate sample standard curves, a global robust value of E can be estimated from the common slope.

In Figure 2A, the dilution-replicate sample standard curves from the myocyte data was fit by constraining the slopes of all samples to be identical. Each sample was also fit independently (regression line not shown) and compared with the constrained fit, with each sample weighted equally in the estimate of E for...
fits is 1.89 and from the constrained fit is 1.82, confirming that the constrained fit approach is less influenced by individual Cq values than the independent fit approach. Moreover, the means of the independent fits approach was not significantly different from that of the constrained fit approach, suggesting that both methods (Fig. 2B). From the plots, one of the GAPDH control samples (solid squares) showed considerable variation. An independent fit of this sample yielded an extreme E of 4.43, which is higher than the average of 2.20 and the constrained fit of 1.95. If this sample is excluded, the average E from the independent fits is 1.89 and from the constrained fit is 1.82, confirming that the constrained fit approach is less influenced by individual Cq values than the independent fit approach. Moreover, the means of the independent fits approach was not significantly different from that of the constrained fit approach, suggesting that

Figure 2. Estimating qPCR efficiency. (A) Cq vs. log(1/Dilution) plots. Data were fit either globally with the constraint that all slopes are identical (shown) or independently (not shown) for each dilution-replicate sample. (B) Comparison of the slopes determined from constrained and independent fit methods. Error bars represent SE of the slopes from the independent fits and SE from the constrained fit, respectively, from n = 4 independent biological samples (different rat litters). None of the efficiencies was statistically different, as determined by an unpaired t-test on their means and standard errors (SE) with n = 4 (independent myocyte isolations).
both estimates are similar and reflect the efficiency of the PCR reactions.

**Estimate of relative changes in gene expression from Cq-Cq plots.** Most qPCR studies involve the quantification of a gene relative to a reference gene. As an extension of the dilution-replicate design strategy, we developed an analytical approach to determine relative gene expression values by using a Cq-Cq plot. Assuming that for each sample the dilutions (d) and threshold (T) quantities used in Cq evaluation are identical for both genes, cancelling out d and taking the log of the right side in Equation 2,

\[
\log[Q_r(0)] + C_{qr} \times \log(E_r) = \log[Q_g(0)] + C_{qg} \times \log(E_g)
\]

where 'r' is the reference gene and 'g' is the target gene of interest. Isolating for C_{qg},

\[
C_{qg} = \frac{\log[Q_g(0)] - \log[Q_r(0)]}{\log(E_g) - \log(E_r)}
\]

**Figure 3.** Cq-Cq Plots and change in relative gene expression ratio. (A) Cq-Cq plots of gene of interest (ERK2/MAPK1 or ANF) vs. reference gene (GAPDH or Rps16). Data were fit either globally with the constraint that all slopes are identical (shown) or independently (not shown) for each sample. (B) Change in relative gene expression ratio for all samples. Ratios were determined by applying the fit-estimated Y-intercept from Cq-Cq plots and the estimated slope from the reference gene Cq-log(1/dilution) plots to Equation 5. "Independent" refers to results based on the independent fits of both Cq-Cq and Cq-log(1/dilution) plots, and "Constrained" refers to results based on the constrained fits. Using Grubb’s outlier test, a statistically significant biological outlier was determined and represented as solid symbols in (B).
(5) $C_{q_g} = \log(E_g)/\log(E_r) \times C_{q_r} - \log(Q_{g(0)}/Q_{r(0)})/\log(E_r)$

With both sample-invariant PCR efficiencies ($E_s$) and initial quantities ($Q(0)$) as constant, Equation 5 describes a linear relationship between the two $C_q$ values. Therefore, a $C_{q_g}$ vs. $C_{q_r}$ plot describes a line with a y-intercept that indirectly corresponds to $Q_{g(0)}/Q_{r(0)}$, the ratio of expression between the two genes (Fig. 1B). An increase in the ratio between the gene of interest ($g$) and a reference gene ($r$) results in a downward shift of the line, whereas a decrease in the ratio results in an upward shift. Similar to the standard curve described above, the y-intercept of the $C_q$-Csq plot can be used to directly and statistically compare relative gene expressions between samples. Further, this ratio can be quantified by using Equation 5 and an estimate of the PCR efficiency of the gene of interest ($g$) from standard curves. As with the standard curve fitting described above, the dilution-replicate experimental design also allows us to estimate the relative gene expression ratio by either a constrained (global) or independent fit of the $C_q$-Csq plots, the latter of which only assumes $E$ to be constant between dilutions and not samples.

We examine $C_q$-$C_q$ plots for the myocyte data in Figure 3A. When $C_q$ values from the PE-invariant ERK2/MAPK1 gene are plotted against a reference gene (GAPDH or Rps16), the points for both control and PE-treated myocytes overlap tightly. In contrast, plots of the ANF data against either reference gene consistently show a downward shift for PE-treated myocytes overlapping with controls, consistent with the fact that ANF gene expression is upregulated by PE.

The $C_q$-$C_q$ plot presented additional benefits, both expected and unexpected. In the standard curve plots, one of the control myocyte samples appeared to contain a relatively low initial quantity of template (solid circles; Fig. 2A). In the $C_q$-$C_q$ plot, this sample was indistinguishable from the other control samples, as expected since the plot represents the relative abundance of two genes. In the standard curve plots, one of the control myocyte samples exhibited a large variation around the linear fit, particularly for GAPDH (solid squares; Fig. 2A). Remarkably, even though this sample also showed large variation with the GAPDH gene, it averaged out in the range of the other samples in the $C_q$-$C_q$ plots.

To quantify the change in relative gene expression between samples, we estimated the y-intercept from the $C_q$-$C_q$ plots for each sample and estimates of $E$ from the standard curve plots are required. Since the slope of the $C_q$-$C_q$ plots is proportional to the relative PCR efficiencies of the genes, assuming that these values are indiscriminately invariant between reactions, the data can be fit independently or by constraining the slopes as the standard curve fitting described above.

We found that the constrained (identical slope) fit gave more consistent results compared with the independent fit of each sample. When both dilution-replicate standard curves and $C_q$-$C_q$ plots were independently fit, the resultant estimated changes in relative gene expression by PE-treatment were sometimes highly variable for two of the samples (square and circle) for both reference genes (Fig. 3B). This large variance was primarily due to individual points in the $C_q$-$C_q$ plots that skewed the fits (Fig. 3A; for ANF vs. Rps16, a single open circle; for ERK2/MAPK1 vs. GAPDH, the obvious solid squares and the inconspicuous solid circles). Remarkably, these large variations appeared to be effectively negated by the constrained fitting routine, resulting in similar estimated changes in relative gene expression with exception to a single sample (solid upward triangles), which did not appear affected by the PE-treatment. Aside from this sample, chronic PE treatment increased ANF gene expression from 10 to 100-fold, consistent with previous reports.8

We evaluated whether specific samples were in fact biological outliers based on the results of the $C_q$-$C_q$ plots. From the data, there appeared to be a single sample where ANF expression appeared indifferent to PE-treatment regardless of the reference gene used (solid upward triangles in Fig. 3B). Using Grubb’s test on the log-transformed changes in expression ratios, the ANF expression of this sample appeared to be significantly lower in the PE-treated myocytes as compared with the other samples only when the constrained fit data are used ($p < 0.05$). This may be due to the large variation in the independent fit data, which attests to the fact that outlier identification is especially difficult with few samples. None of the other determined changes in expression ratio were found to be significant outliers regardless of fitting routine used, reinforcing the notion that the constrained fit provides more robust and consistent results than the independent fits.

Detection of small changes in gene expression. The greatest strength of qPCR is its sensitivity and capacity to detect subtle changes. However, the general practice is that the ability of detecting subtle changes in the order of 2-fold requires very skilled hands and accurate machines. We next tested if our experimental design strategy was sensitive, yet robust enough to detect smaller changes in gene levels of ion channels, which are usually expressed in low abundance. Reports from both functional11-14 and gene expression studies15,16 suggest that high- (CACNA1C) and low- (CACNA1G) voltage-dependent calcium channels are enhanced in hypertrophic and/or PE-treated cardiomyocytes compared with control.17 In contrast to ANF, the increases of the calcium channels are only around a few folds, making them suitable to test our novel qPCR strategy. As shown in Figure 4, we evaluated the gene expression levels of CACNA1C (GenBank no. NM_031601) and CACNA1G (GenBank no. NM_012517) in PE-treated ventricular myocytes. The standard curves for the three genes were typical linear and generally tight between most samples (Fig. 4A), with three important exceptions. First, a single control sample (solid squares) has $C_q$ values larger than the other samples for all the genes, indicating that it contained much less DNA than the other samples (the same sample used in the ANF experiments). Second, the $C_q$ values for CACNA1C are more variable than expected. Lastly, a single PE-treated sample (open up-triangle) displayed consistently lower $C_q$ values than the other samples only for CACNA1G, indicating that this sample expressed more of that gene.

When we determined the efficiencies from the standard curves, both via independent and slope-constrained fits, there results between the two methods were not significantly different (Fig. 4C). Although the control sample with lower CACNA1C expression had a large impact on the estimate of PCR efficiency using the independent fits method, it did not impact the results.
potentially more problematic to discern from noise that may arise from the various processes during the qPCR setup and experimentation. Even so, the Cq-Cq plot of CACNA1G still revealed a generally lower set of Cq data for the PE-treated group than for the control. After applying the efficiency factor, we determined that the expression of CACNA1G was enhanced by PE regardless from the constrained fit approach (both also had large standard errors resulting from this sample).

Using Cq-Cq plots, we estimated the changes in expression ratio for both calcium channel genes relative to β-actin (Fig. 4B). The data were more tightly clustered than for our ANF results, indicating that the changes in gene expression are smaller and potentially more problematic to discern from noise that may arise from the various processes during the qPCR setup and experimentation. Even so, the Cq-Cq plot of CACNA1G still revealed a generally lower set of Cq data for the PE-treated group than for the control. After applying the efficiency factor, we determined that the expression of CACNA1G was enhanced by PE regardless
of the fit routine, with one exception (Fig. 4D). The exception comes from the sample which showed the highest Cq values, and therefore smallest amount of DNA. Using Grubb’s test, the independent-fit of this sample (solid circle, Fig. 4D), was determined to be significantly smaller than the other three samples. Remarkably, even with the low DNA concentration, the relative change in gene expression level determined from the constrained-fit for this sample was consistent with that for the other four samples. Moreover, spread in the relative gene expression values was smaller for the constrained-fit (1.77 - 4.37× relative to control) vs. the independent-fit (0.39 - 9.55× relative to control) results. The change in CACNA1G expression is consistent with previous reports.17

The results for CACNA1C were even tighter that any difference was very difficult to visually determine even in with the Cq-Cq plots (Fig. 4B). In the insets for this plot, the independent (upper inset), and constrained (lower inset) fits for the first two pairs of samples (squares and circles), are shown to visually describe how the two methods can result in different conclusions. Notably, the independent-fits are more variable. As shown in Figure 4D, the poorest sample (solid circle) resulted in a poorly estimated 42,331-fold increase in the relative expression of CACNA1C. Grubb’s test indicates that this sample is an outlier compared with the other three samples. In contrast, the constrained-fit appeared to describe the data with a greater consistence, even for the poorest sample, estimating it at 2.1-fold times the control. Overall, PE appeared to increase the expression of CACNA1C by 100%, as determined by both fitting methods, consistent with previous reports.12,15

Taken together, our method was effective in discerning small changes in gene expression for low-expression of ion channel genes. Even with problematic data, the constrained-fit routine gave tighter and more consistent results compared with independent fits. More importantly, the use of our dilution-replicate design not only allowed us to evaluate the quality of each qPCR reaction (via consistency in the standard curve and the Cq-Cq plots), but also to effectively estimate the reaction efficiency using only three dilution points and constraining the fits.

**Discussion**

Real-time PCR is a highly-sensitive and popular approach to quantifying gene expression. Yet this sensitivity is also its shortcoming. To minimize variations in qPCR results, a consortium of researchers developed the MIQE guidelines detailing how experiments should be performed and documented.1 However, adherence to all aspects of the guidelines can be limited by cost and practicality, and may not always improve the results. The new experimental design in our study addresses these limitations. Specifically, rather than performing identical technical replicates and constructing independent sample standard curves, our design proposes the use of dilution-replicates which are not only used to construct standard curves but also used to estimate gene expression levels, thus not only improves the robustness of the results but also requires fewer reactions than the traditional approach, thereby reducing costs. Because the PCR efficiency (E) and initial gene quantity are measured for every test sample, we can either analyze each independently or collectively (i.e., PCR efficiency can be either variable or constant between samples). This experimental design strategy helps to minimize the effects of sample noise from technical variance and identify problematic results, and should be used in conjunction with the MIQE guidelines.

Technical noise (e.g., pipetting errors, differences between plates, noise in qPCR system, time of day, etc.) alone can amount to substantial variations in the results. In a study where only a single source of DNA template was used, Cq values from intra-run and inter-run reactions were found to vary by up to 0.367 units and 0.395, respectively, and PCR efficiency (E) estimates by up to 0.03 units.18 Understandably, this means more replications of the experiment should be performed to lower the variation until a stable mean Cq and PCR efficiency (E) can be determined. However, the traditional method of using 2–3 replicates of a sample (and standard curves) can be impractical and costly when a large number of genes are examined. Because the largest variation in PCR efficiency (E) comes from the differences in samples, how the samples are prepared, and the quality of the preparation, it is more reliable to estimate PCR efficiency (E) from as many independent standard curves as possible.

To devise a practical approach that maximizes the use of all samples in a qPCR study, we focused on the qPCR steps in estimating PCR efficiency (E) and Cq values from all samples. Our dilution-replicate design strategy determines E from all the samples, so more samples lead to more accurate estimate of PCR efficiency (E). Furthermore, whereas the traditional design strategy requires that all sample Cq values lie within the linear dynamic range of the independent sample standard curve, the dilution-replicate design strategy provides a dynamic range for each sample due to its own standard curve. Specifically, if a Cq value determined from the traditional design method lies outside the linear dynamic range of a standard curve, the qPCR must be redone using a different dilution. In contrast, because the dilution-replicate design method determines Cq values at multiple dilutions of each sample, there is a lower chance that none of the Cq values overlaps with that of other samples. Moreover, even if there is no overlap in the sample Cq values, the consistency in PCR efficiency (E) can still be evaluated by comparing the standard curves between samples.

As a rule of thumb, we recommend first estimating the lowest suitable dilution by running a standard 30-cycle PCR on the highest expressing gene in the study (usually the reference gene). The amplicon band should be a very high intensity sharp band on an agarose gel, but not saturated. For the qPCR reactions, this value should be the starting point and serial dilutions be applied accordingly such as 5 or 10, ensuring that the reaction does not prematurely saturate for any of the genes. For more dilution points, such as 5 that is used in the tradition standard curve, 2- to 5-fold serial dilutions have been used. We emphasize that in contrast to the traditional qPCR experimental design, our dilution-replicate design does not require sample Cq values to overlap. Instead, the consistency in PCR efficiency is determined by examination of each sample standard curve.
Table 1. Primers used in qPCR experiments. Primers are listed 5' → 3'

| Gene                  | Forward primer         | Reverse primer         | Amplicon |
|-----------------------|------------------------|------------------------|----------|
| GAPDH (NM_017008)     | GGC AAG TTC AAC GGC ACA G | CGC CAG TAG ACT CCA CGA CAT | 142 bp   |
| Rps16 (NM_001169146)  | TAC TTG TAG CTA TGC GGT CCA | CGC GGC TGC ATC ATC T | 145 bp   |
| ANF (M27498)          | TTT CAA GAA CCT GCT AGA CCA | CCT CCA TCT CTC TGA GGC G | 168 bp   |
| ERK2/MAPK1 (NM_053842)| GTT CCC AAA CGA TGA CTC C | GTA AGT CGT CCA GCT CCA TGT C | 182 bp   |
| β-Actin (NM_031144)   | TGC TAT GTT GCC CTA GAC TTC | ATC GGA ACC GCT CAT TG | 104 bp   |
| CACNA1C (NM_012517)   | ATG GTC TCT CTC AGG AGT TGG CCG | TGC AAA TGT GGA ACC GGT GAA GTG | 135 bp   |
| CACNA1G (NM_031601)   | TCA CGC AGC TCA ACG ACC TGT CC | GGC TGT CTC GGC TCA AGT AGA AG | 150 bp   |

The PCR amplification reaction is sensitive to impurities\(^9\) such as from the DNA purification procedure, including detergents (SDS),\(^20\) ethanol and isopropanol\(^21\) and phenol,\(^22\) so the efficiency cannot always be reliably deemed sample-independent without just evaluation. For this reason MIQE guidelines recommend pre-testing the quality of the sample prior to the qPCR experiment. However, some of these contaminants are not easily detectable, resulting in concentration-dependent effects on the PCR efficiency that can result in potentially large errors in the qPCR results. In the efficiency correction and ΔΔCt design strategies, the PCR efficiency is assumed invariant between samples so this anomaly cannot be deduced from the test samples, which are performed only as identical replicates. In contrast, this anomaly can be easily observed by using the dilution-replicate design strategy, where the PCR efficiency can be evaluated and compared using independent fits of each sample standard curve.

Some studies offer methods which do not rely on sample invariant PCR efficiencies. In these, sample efficiencies and initial gene quantities were estimated from the amplification (fluorescence) curve by a model-based paradigm\(^23,24\) or linear fit of the exponential phase.\(^25\) The former has been criticized for relying on a clear robust signal in the early PCR cycles to be able to accurately estimate the initial gene quantity;\(^26\) and both methods measure efficiencies on a sample-by-sample basis, requiring more extensive analysis, though doable, not currently easily amenable to common existing commercial qPCR packages (e.g., Sequencing Detection System and Light Cycler software). Because our dilution-replicate experimental design approach extracts the same data (Cq values) as the efficiency correction approach and also collects data on sample-independent PCR efficiencies, we believe it offers a balance between the traditional and non-invariant efficiency methods.

The major benefit of the dilution-replicate design strategy is that it yields data that simultaneously measures both PCR efficiency and DNA quantity for all samples. First, this results in fewer reactions than the traditional efficiency correction method. Second, the PCR efficiency can be estimated from either a constrained global fit or independent fits of each sample, the latter of which can be used to assess the invariability of the PCR efficiency across samples and identify anomalous samples which may contain PCR inhibitors, an issue that cannot be evaluated by either the efficiency correction or ΔΔCq methods without more experiments. Third, the dilution-replicate design strategy allows for an easy visual evaluation of the relative change in expression of two genes via Cq-Cq plots independently of the PCR efficiency, in contrast with the efficiency correction method that requires evaluating an expression involving the Cq and E values for both genes and samples.\(^2\) In conclusion, the dilution-replicate experimental design strategy is a simple, more effective and more robust strategy to real-time PCR gene expression studies than current approaches, especially for ion channel research.

Materials and Methods

Ventricular myocyte isolation and cell culture. Sprague-Dawley rat pups (Charles River Laboratories) were used in this study. Animal care and use procedures were approved by the Institutional Animal Care and Use Committee of the University of Toronto. The pups (1 d old) were anaesthetized by isoflurane prior to any surgical procedure. As previous described,\(^27,28\) myocytes were dissociated from ventricles by gentle agitation in Hanks buffer containing Trypsin/collagenase and cultured at a confluent density of 10\(^6\) cells/35 mm dish in 5% FBS media (1:1 DMEM:F12, 1% Penicillin-Streptomycin) for 24 h. The culture medium was then replaced with fresh serum-free media (1:1 DMEM:F12, 1% Penicillin-Streptomycin, 1% ITS, 100 μM BrDU, 1nM LiCl, 25 μg/ml ascorbic acid). The dishes were divided into two groups, the mock control (water treatment) and phenylephrine (10 μM PE, Sigma-Aldrich, in 0.01% DMSO, Fisher Scientific), and cultured for four additional days. After four days, myocytes from the PE-treated dishes were confirmed notably larger than from the mock control, a typical visual sign of hypertrophy. Each set of the myocyte isolations comprised a single sample in this study.

RNA isolation and cDNA preparation. Total RNA was isolated from each dish of cultured rat neonatal ventricular myocytes using 200 μl TriReagent (Sigma-Aldrich) following the manufacturer’s protocol. Because of the small quantity of sample (particularly for low expressing genes), the total RNA was resuspended in 14 μl nuclelease-free water (Qiagen) and the entire sample was used to synthesize cDNA by combining it in a fresh tube with 1 μl M-MLV reverse transcriptase (Sigma-Aldrich), 2 μl RT buffer, 1 μl RNaseOUT (Life Technologies), 1 μl of 10 mM dNTP (Qiagen), and 1 μl of 0.4 μg/μl oligo-dT primer (Qiagen). The reaction was incubated at 37°C for 1 h and then terminated by heat inactivation at 80°C for 10 min. To remove the RNA, 1 μl RNase H (Life Technologies) was added and then incubated at 37°C for 20 min. The resultant cDNA was subsequently purified by running the entire sample through a
QIAquick nucleotide removal column following the manufacturer’s protocol, and doubly eluted with 30 μl nuclease-free water (Qiagen). The cDNA sample was kept at −20°C until use.

Quantitative real-time PCR. Platinum SYBR Green qPCRSuperMix-UDG with ROX (Life Technologies) was used in the qPCR step. For simplicity of pipetting, master mixes were prepared: one for each primer step (with the SuperMix) and one for each dilution of cDNA sample [using 0.5% diethyl pyrocarbonate (Sigma Aldrich)-treated water], the latter of which was performed serially. For example, for four primer pairs, four cDNA samples and three cDNA dilutions, there were 4 × 4 × 3 master mixes, each of which was added to the appropriated reaction wells independently. Effectively, each 10 μl qPCR reaction contained 5 μl of the SuperMix, 1 μl of each primer (0.5 μM final concentration) and a diluted sample of cDNA. The master mixes were combined in the wells of 38-well micro-well plates and run on an Applied Biosystems (ABI) 7900HT Real-Time PCR System controlled by SDS2.2.1 software using the following program: 95°C/5 min, 40 cycles of (95°C/30 s, 55°C/1 min), followed by a melting curve. The peak of the amplification curve was performed serially. For example, for four primer pairs, four cDNA samples and three cDNA dilutions, there were 4 + 4 × 40 cycles. Primers and target amplicon length are shown in Table 1. All primers were ordered from IDT DNA and re-suspended in nuclease-free water (IDT).

Data analysis. Amplification curves were baseline-subtracted using the average of a 5-cycle range in the flat region just prior to the exponential region of the curve. Cycle-threshold (Cq) values were determined in SDS2.2.1 software using a fixed threshold of 0.2. This threshold value appeared suitable for all amplification curves acquired in this study, lying within the early phase of the exponential region. The resultant Cq values were subsequently plot in OriginPro 8 (OriginLab) to determine primer PCR efficiencies and gene quantities as described in the results. Where described, outlier samples were identified by Grubb’s test22 with p-values estimated using the Outlier package available in R.30

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
1. Bustin SA, Benes V, Garson JA, Hellmann J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009; 55:611-22; PMID:19246619; http://dx.doi.org/10.1373/clinchem.2008.112797
2. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45; PMID:11308866; http://dx.doi.org/10.1093/nar/29.9.e45
3. Simpson P. Stimulation of hypertrophy of cultured neonatal rat heart cells through an alpha 1-adrenergic receptor and induction of beating through an alpha 1- and beta 1-adrenergic receptor interaction. Evidence for independent regulation of growth and beating. Circ Res 1985; 56:884-94; PMID:2988814; http://dx.doi.org/10.1161/01.RES.56.6.884
4. Simpson PC, Kariya K, Karns LR, Long CS, Kallner JS. Adrenergic hormones and control of cardiac myocyte growth. Mol Cell Biochem 1991; 104:43-53; PMID:1656195; http://dx.doi.org/10.1007/BF00229801
5. Knowlton KU, Baracchini E, Ross RS, Harris AN, Henderson SA, Evans SM, et al. Co-regulation of the atrial natriuretic factor and cardiac myosin light chain 2 genes during alpha-adrenergic stimulation of neonatal rat ventricular cells. Identification of cis sequences within an embryonic and a constitutive contractile protein gene which mediate inducible expression. J Biol Chem 1991; 266:7759-68; PMID:1850419
6. Barton AJ, Finn SG, Fuller SJ. Chonic activation of extracellular-signal-regulated protein kinases by phenylephrine is required to elicit a hypertrophic response in cardiac myocytes. Biochem J 2003; 371:71-9; PMID:12513666; http://dx.doi.org/10.1042/BJ20021395
7. Somura F, Izawa H, Iwase M, Taleichi Y, Ishiki R, Nishizawa T, et al. Reduced myocardial sarcoplasmic reticulum Ca(2+)-ATPase mRNA expression and biphasic force-frequency relations in patients with hypertrophic cardiomyopathy. Circulation 2001; 104:658-63; PMID:11489771; http://dx.doi.org/10.1161/01.HC3101.093869
8. Winer J, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 1999; 270:41-9; PMID:10328763; http://dx.doi.org/10.1006/abio.1999.0485
9. Bouzaghrane F, Reinhardt DP, Reudelhuber TL, Thibault G. Enhanced expression of fibrillin-1, a constituent of the myocardial extracellular matrix in fibrosis. Am J Pathol Heart Circ Physiol 2005; 289:H982-91; PMID:15889235; http://dx.doi.org/10.1152/ajpheart.00151.2005
10. Iglarz M, Touyz RM, Viel EC, Paradis P, Amiri F, Diep QN, et al. Peroxisome proliferator-activated receptor-alpha and receptor-gamma activators prevent cardiac fibrosis in mineralocorticoid-dependent hypertension. Hypertension 2002; 39:47-53; PMID:12868386; http://dx.doi.org/10.1161/01.HI.0000083511.91817.B1
11. Zhang S, Hiraoa M, Hirano Y. Effects of alpha1-adrenergic stimulation on L-type Ca2+ current in rat ventricular myocytes. J Mol Cell Cardiol 1998; 30:1995-65; PMID:9799650; http://dx.doi.org/10.1006/jmcc.1998.0758
12. Liu QY, Karpinski E, Fang PK. The L-type calcium channel current is increased by alpha-1 adrenoceptor activation in neonatal rat ventricular cells. J Pharmaco Exp Ther 1999; 291:43-53; PMID:9765815
13. Nuss HB, Houser SR. T-type Ca2+ current is expressed in hypertrophied adult feline left ventricular myocytes. J Mol Cell Cardiol 1998; 30:1955-65; PMID:9799650; http://dx.doi.org/10.1006/jmcc.1998.0758
14. Martínez ML, Heredia MP, Delgado C. Expression of T-type Ca2+ channels in ventricular cells from hypertrophied rat hearts. J Mol Cell Cardiol 1999; 31:1617-25; PMID:10473146; http://dx.doi.org/10.1016/S0022-5191(99)90098-9
15. Fan JQ, Chen B, Marsh JD. Transcriptional regulation of T-type calcium channel expression in cardiac myocytes. J Mol Cell Cardiol 2000; 32:1841-9; PMID:11013128; http://dx.doi.org/10.1006/jmcc.2000.1217
16. Haase H, Kresse A, Holhaus A, Schulte HD, Maier M, Osterziel KJ, et al. Expression of calcium channel subunits in the normal and diseased human myocardium. J Mol Med (Berl) 1996; 74:99-104; PMID:8820405; http://dx.doi.org/10.1007/BF00196785
17. Cribs L. T-type calcium channel expression and function in the diseased heart. Channels (Austin) 2010; 4:47-52; PMID:21139421; http://dx.doi.org/10.4161/chann.6.4.12870
18. Rulledge RG, Coté C. Mathematics of kinetic PCR and the application of standard curves. Nucleic Acids Res 2003; 31:493; PMID:12907775; http://dx.doi.org/10.1093/nar/gng093
19. Rådström P, Knutsson R, Wolffs P, Lövenklev M, Löfström U, Troux M, et al. Pre-PCR processing: strategies to generate PCR-compatible samples. Mol Biotechnol 2004; 26:133-46; PMID:14764939; http://dx.doi.org/10.1385/MB:26:2:133
20. Weyant RS, Edmonds P, Swaminathan B. Effect of ionic and nonionic detergents on the Taq polymerase. Biotechniques 1990; 9:308-9; PMID:2220370
21. Loffert D, Stump S, Schaffrath N, Berkenkopf M, Kang J. PCR: Effects of template quality. Quanar News 1997; 1:8-10
22. Katcher HL, Schwartz I. A distinctive property of Tn5 DNA polymerase: enzymatic amplification in the presence of phenol. Biotechniques 1994; 16:84-92; PMID:8136148
23. Liu W, Saint DA. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal Biochem 2002; 302:52-9; PMID:11846375; http://dx.doi.org/10.1006/abio.2001.5530
24. Rulledge RG. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. Nucleic Acids Res 2004; 32:e178; PMID:15601990; http://dx.doi.org/10.1093/nar/gnh177
25. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 2003; 339:62-6; PMID:12618301; http://dx.doi.org/10.1016/S0304-3940(02)01425-4

26. Swillens S, Desaies B, Housni HE. Revisiting the sigmoidal curve fitting applied to quantitative real-time PCR data. Anal Biochem 2008; 373:370-6; PMID:17996715; http://dx.doi.org/10.1016/j.ab.2007.10.019

27. Gong N, Bodi I, Zobel C, Schwartz A, Molkentin JD, Backx PH. Calcineurin increases cardiac transient outward K+ currents via transcriptional up-regulation of Kv4.2 channel subunits. J Biol Chem 2006; 281:38498-506; PMID:17060317; http://dx.doi.org/10.1074/jbc.M607774200

28. Wang L, Feng ZP, Kondo CS, Sheldon RS, Duff HJ. Developmental changes in the delayed rectifier K+ channels in mouse heart. Circ Res 1996; 79:79-85; PMID:8925572; http://dx.doi.org/10.1161/01.RES.79.1.79.

29. Grubbs FE. Procedures for detecting outlying observations in samples. Technometrics 1969; 11: 1-21; http://web.ipc.caltech.edu/staff/fmasci/home/statistics_refs/OutlierProc_1969.pdf

30. Komsta L. Processing data for outliers. R Journal 2006; 6: 10-3; http://www.r-project.org/doc/Rnews/Rnews_2006-2.pdf