Reference Gene Validation for Quantitative Real-time PCR Studies in Amphibian Kidney-derived A6 Epithelial Cells

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Abstract

Quantitative real-time PCR (qRT-PCR) is a widely used technique that relies on reference genes for the normalisation of gene expression. These reference genes are constitutively expressed and must remain stable across all samples and treatments. Stability of housekeeping genes may vary and must be optimised for a specific tissue, sample or cell line. We here present a study screening for possible reference gene candidates, *eef1a1*, *rpl8*, *sub1.L*, *clta*, *H4* and *odc1*, in the *Xenopus laevis* (A6) kidney cell line. Quantification cycle results were analysed by using geNorm, to calculate the average expression stability and the coefficient of variation for each candidate reference gene. All of the tested genes met the guidelines for stable reference genes, namely an average expression stability of < 0.5 and a coefficient of variation value of < 0.2, with *eef1a1* > *sub1.L* > *rpl8* > *clta* > *odc1* > *H4*. By using pairwise variation analysis, the optimal number of reference targets was determined to be 2. As such, we report that the reference genes *eef1a1* and *sub1.L* should be used to achieve optimal normalisation in A6 cells.

Keywords

A6 cells, *in vitro*, reference genes, qRT-PCR

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Introduction

Quantitative real-time PCR (qRT-PCR) is a commonly-used technique to measure gene expression in a wide variety of samples and tissues from a variety of species. However, stable reference genes should be selected in order to obtain reliable results.[1] Many molecular analyses still contain quantitative PCR data that are poorly normalised. However, in 2009, the MIQE (**M**inimum **I**nformation for Publication of **Q**uantitative Real-time PCR **E**xperiments) Guidelines were published, providing an overview of the essential criteria required for publication of quantitative PCR data, including the selection of the most stable reference genes.[2] Reference gene mRNA expression should be stable, meaning that there should be very little variation in expression across different samples, and its abundance should be in direct correlation with the total amount of mRNA in the sample.[2] The use of a single reference gene for normalisation is considered unacceptable, and the optimal number of choice must be experimentally determined. Mathematical algorithms, such as geNorm analysis, are widely used to determine the most stable reference genes and the optimal number that should be used with a given sample set.[3]

Xenopus laevis or the African clawed frog, is a widely used laboratory animal. Around 1930, this amphibian species became routinely used in a pregnancy test known as the 'Hogben test'.[4] As a result, *Xenopus laevis* became commonplace in European and North American laboratories.[4] The ability to reliably obtain eggs, throughout all seasons, made *Xenopus laevis* a valuable laboratory animal for use in the field of developmental biology. With the genome now being fully sequenced, this species has more recently been widely used for various genetic approaches and as a model for the study of a number of human diseases.[5–6] However, *in vitro* experiments with amphibian cell lines, such as the immortalised *Xenopus laevis* A6 kidney cell line, could provide an alternative to *in vivo* experiments, thus reducing the number of laboratory animals used.[7] A6 cells have already been proven useful in gene expression studies,[8–14] and these cells are particularly of interest as an *in vitro* infection tool in ranavirus research.[10, 14–15] However, a thorough screening for reliable reference genes is still lacking.

In this study, we examined the stability of a number of traditionally-used and alternative reference genes in A6 cells. Classic reference genes are mostly orthologues of genes found to be stably expressed in mammalian tissues. In the current study, these included: elongation factor eEF-1 alpha (*eef1a1*); ribosomal protein L8 (*rpl8*); histone H4 (*H4*); and ornithine decarboxylase (*odc1*).[16–18] Alternative reference genes analysed in this study included: SUB1 homologue (*sub1*); and clathrin light chain A (*clta*). By using RNA-sequencing, these genes were previously identified as remaining relatively stable during *Xenopus laevis* development.[18]

Materials and Methods [L1]

In vitro culture of A6 cells [L2]

A6 cells were grown in 75 cm^2 cell culture flasks (Greiner bio-One, Vilvoorde, Belgium) in A6 medium (74% (v/v) NCTC 109 medium, 15% (v/v) distilled water, 10% (v/v) fetal bovine serum (FBS), 1% (v/v) of a 10,000 U/ml penicillin–streptomycin solution). Cells were incubated at 26°C, 5% (v/v) CO_2 until they reached confluence. The cells (maximum passage 7) were detached by trypsinisation and subcultured in 6-well plates (Greiner bio-One), at a density of 3 x 10^5 cells per well in 3 ml A6 medium. After 24 hours, the confluency reached about 90% (late log-phase/early stationary-phase). The cells were washed with HBSS+ and incubated with A6 medium alone, or with A6 medium supplemented with tryptophol (1 μM, 100 μM or 1 mM), for 24 hours.[19] Tryptophol is a metabolite produced by, *inter alia*, amphibian skin bacteria[20] and the amphibian fungal skin pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*.[19] Each experimental condition was tested with six biological replicates.

RNA extraction and cDNA synthesis [L2]

Total RNA was isolated from the A6 cells by using the RNeasy mini kit (Qiagen, Antwerp, Belgium). Briefly, the cells were treated with 350 μl RLT buffer containing 35 μl β-mercaptoethanol. To obtain efficient cell lysis and homogenisation, samples were transferred to an eppendorf tube containing six 2.3 mm silica beads and the tubes were placed in a Qiagen TissueLyser II (3 × 1.5 minutes at 30 Hz, with 30 seconds between each burst, during which time the samples were maintained on ice). After centrifugation at 15,870*g* for 1 minute, the lysate supernatant was mixed with an equal volume of 70% (v/v) ethanol and loaded onto an RNeasy mini spin column (Qiagen). The columns were

centrifuged for 1 minute at 15,870*g*, the flow through discarded and the column then washed with 350 µl RW1 buffer. Subsequently, the column was treated with DNAse I (Qiagen) for 15 minutes at room temperature, washed with 350 µl RW1 buffer and thereafter washed twice with 500 µl RPE buffer. Finally, the RNA was eluted from the column by adding 30 µl RNAse-free water. The RNA concentration was measured by determining the absorbance at 260 nm with a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the quality of the RNA was checked by using an Experion RNA StdSens Analysis Kit (Bio-Rad, Temse, Belgium). Total RNA (1 µg) was reverse transcribed to cDNA with a iScript cDNA Synthesis Kit (Bio-Rad) and cDNA was stored at –20°C until required.

Primer design [L2]

The primer sequences used for *eef1a1*, *odc1*, *rpl8*, *sub1.L* and *clta.L* were obtained from the published literature.[16–18] Primers for *H4*, *tnfrsf10b.L* and *tnfrsf10b.S* were designed for this study by using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 1).[21] The specificity of each primer set was checked by Nucleotide BLAST (Nucleotide Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and by performing a standard PCR (40 cycles) on a cDNA mixture of all samples (diluted 1:5) followed by gel electrophoresis. The PCR products were checked on an agarose gel (1.5% w/v) and single band amplification was confirmed (Figure 1). Primer efficiency was evaluated by using serial dilutions of the cDNA sample mixture (1:5, 1:25, 1:125; 1:625). For every standard curve, we assessed the amplicon efficiency (E), correlation coefficients (R^2) and slope (Table 1). Water and no-template controls were used as negative controls for each primer set. The melting curves were also analysed, and for all primer pairs a single peak was detected.

qRT-PCR analysis and data analysis [L2]

Quantitative real-time PCR reactions were run in duplicate. The 10 µl reaction mixture consisted of: 5 µl iQ SYBR Green Supermix (Bio-Rad); 4 µl HPLC-grade water (Merck Millipore, Overijse, Belgium) containing 1.25 µM each of the forward and reverse primers; and 1 µl 1:5 diluted cDNA. The PCR protocol (40 cycles) was performed on a CFX384 Touch™ Real-Time PCR System with a C1000 Thermal Cycler (Bio-Rad). The results were analysed by using the Bio-Rad CFX manager 3.1. Quantification cycle (Cq) values were obtained using auto baseline settings, and these were applied per primer set. These raw Cq values were imported in QBase for fully automated analysis and interpretation of reference gene stability using the geNorm algorithm.[22]

Expression analysis of target genes [L2]

Tnfrsf10b.L and *tnfrsf10b.S* were used as target genes to analyse the usefulness of the selected candidate reference genes. The results are shown as fold changes of mRNA expression, which were calculated based on the CNRQ (Calibrated Normalised Relative Quantity) values obtained in QBase.[22]

Statistical analysis [L2]

All statistical analyses were performed by using SPSS version 25 (SPSS Inc., Chicago, IL, USA). Normality of the CNRQ data was assessed by using a Kolmogorov–Smirnov and Shapiro–Wilk test, showing a normal distribution. A Levene's test was used to validate the equality of variances and a one-way ANOVA with a Bonferroni post hoc test was used to determine the significance of the differences between mean values, with significance set at $p \leq 0.05$.

Results [L1]

Variation in Cq values of the candidate reference genes [L2]

We investigated the qRT-PCR expression profiles from candidate reference genes in A6 cells and determined the Cq values in untreated A6 cells and tryptophol-treated A6 cells (1 μM, 100 μM and 1 mM), with six biological replicates per experimental condition (Figure 2). There was little variation between the different test conditions, and *eef1a1*, *rpl8* and *sub1.L* showed the least deviation between all the samples. The mean Cq values ± SD of *eef1a1*, *rpl8*, *sub1.L*, *clta*, *H4* and *odc1*, respectively, were: 13.33 ± 0.28; 15.23 ± 0.25; 19.93 ± 0.31; 20.91 ± 0.34; 18.76 ± 0.35; and 17.68 ± 0.52.

Stability analysis of the candidate reference genes [L2]

The stability of the reference genes was assessed by using geNorm analysis. This is one of the most widely used algorithms for determining the most stable genes in a given sample panel.[3] It determines a 'geNorm M' value, which indicates the average expression stability of remaining reference genes when a stepwise exclusion of the least stable reference gene is performed. This means that the higher the geNorm M value is, the lower the stability of the reference gene. A coefficient of variation (CV) value is also calculated as a relative standard deviation. For homogeneous samples, such as cell cultures from the same cell type, the M value should be lower than 0.5 and the CV value should be below 0.2. As shown in Figure 3, all of the candidate reference genes met these conditions. However, *H4*, *odc1* and *clta* could be classified as the least stable, whereas *rpl8*, *sub1* and *eef1a1* were shown to be the most stable, with *sub1.L* and *eef1a1* showing notably high reference target stability (geNorm M < 0.2).

Determining the optimal number of reference genes [L2]

The optimal number of reference genes required for normalisation can be calculated by using geNorm analysis of the pairwise variation 'V' value. This value is an indication of how much difference it makes when using an extra reference gene for normalisation. If the geNorm analysis indicates that there is limited added value (cut-off: V < 0.15), then the inclusion of an additional reference gene is not necessary. The V2/3 value represents the pairwise variation of two genes, as compared to that of three genes. In our analysis, the V2/3 is 0.065, indicating that adding a third reference gene will not have a serious impact on the normalisation, and therefore, is not necessary (Figure 4).

Reference gene validation [L2]

In mammals, it has been proposed that tryptophol induces apoptosis by enhancing the formation of the death-inducing signalling complex including tumor necrosis factor receptor superfamily member 10b (TNFRSF10B). In amphibians, xDR-M1 (*tnfrsf10b.L*) and xDR-M2 (*tnfrsf10b.S*) have been described as members of the death receptor family, encoding TNFRSF10B.[23–25]

We analysed the expression profiles of the target genes, *tnfrsf10b.L* and *tnfrsf10b.R*, and compared different normalisation strategies. Based on the above results, *eef1a1* and *sub1.L* were proposed to be the best combination of reference genes, whereas *H4* was shown to be the least stable reference gene. When normalising with *eef1a1* and *sub1.L*, a significant increase in target gene expression was observed when A6 cells were treated with the highest tryptophol concentration (1 mM) (Figure 5a). This was in line with the data obtained when taking all of the tested reference genes into consideration (Figure 5c). Aberrant conclusions could easily be drawn when normalising with *H4* (Figure 5b), as the data were significant only for *tnfrsf10b.S* in this case, but after 100 μM tryptophol treatment as well as 1 mM treatment. These data indicate that the use of inappropriate reference genes for target gene validation can change the interpretation of the observed expression patterns.

Discussion

It is still too often the case that only one reference gene, or even non-validated reference genes, are used in a qRT-PCR experimental set-up.[26] This, however, can lead to misleading results and therefore it is recommended that between two and five stable reference genes are used. Use of the three best reference genes is also a common strategy, which is of course more reliable than just using one single reference gene.[27] However, with user-friendly software packages available (such as geNorm, NormFinder and BestKeeper[3, 28–29]), a thorough screening can be performed to determine the stability of the reference genes and the optimal number that should be included. By using geNorm, we showed that the inclusion of a third reference gene (*rpl8*) is not required when normalisation is carried out with *eef1a1* and *sub1* in A6 cells. This is most likely due to the high stability of these two particular genes in our model (geNorm stability: $M < 0.2$).

The *eef1a1* gene encodes an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. It is a commonly used reference gene in *Xenopus laevis*,[17] and in other animal species,[30] plants[31] and cell lines.[32] Although it is commonly-used, *eef1a1* should not be generalised as the perfect reference gene. Recently, it was shown that *eef1a1* expression can vary depending on the developmental stage of *Xenopus laevis*, whereas *sub1.L* expression remained relatively stable.[18]

The *sub1* gene encodes a coactivator that functions co-operatively with TATA box binding protein associated factors and mediates functional interactions between upstream activators and the general transcriptional machinery.[18] Although *eef1a1* expression was shown to remain stable during differentiation of human vascular stromal cells into adipocytes,[32] and it has been shown that *sub1* stays stably expressed during different developmental stages of *Xenopus laevis* embryos,[18] it should be taken into account that the stability of the reference genes can vary depending on the growth stage of the A6 cells (e.g. completely differentiated A6 cells versus actively dividing cells).[33]

Treatment of A6 cells with tryptophol had little effect on the stability of the tested reference genes. However, it is likely that other chemicals could have an effect on the stability of these reference genes. A recent study by Mughal et al.[18] investigated the variance in expression profile and stability of 16 possible reference gene candidates, including *eef1a1*, *clta, odc1* and *sub1*, in NF48 tadpole brains after a 3-day exposure to

thyroid hormone (T_3), its antagonist (NH3) and the thyroid signalling-altering chemical Triclosan. Depending on the gene and the test conditions, changes in reference gene stability were observed.[18] As such, our results will be of use to those studying gene expression in A6 cells, but depending on the experimental set-up, researchers should carefully plan the best normalisation strategy.

The availability of stable reference genes make the A6 cell line a good model for gene expression studies that can be applied in addition to, or as a replacement for, *in vivo* experiments. This technique can potentially be used as a (pre) screening method to reducing the number of animals used, or as an alternative to *in vivo* experiments, thus replacing the *Xenopus laevis* animal model. As such, the use of A6 cells in gene expression studies is in line with the Three Rs principles, as defined by Russell and Burch.[34]

Conclusions

We screened six reference genes (*eef1a1*, *rpl8*, *sub1.L*, *clta*, *H4* and *odc1*) that have been described for the normalisation of genes in *Xenopus laevis* tissue, to determine whether they are suitable for use as reference genes in the *in vitro* amphibian A6 cell line. By using geNorm analysis, we identified *eef1a1* and *sub1.L* as the most stable genes, with their geNorm M values of < 0.2 and CV values of < 0.2 indicating a very high reference target stability. The optimal number of reference targets was shown to be two. As such, to achieve optimal normalisation in A6 cells, we suggest that *sub1* and *eef1a1* should be used as reference genes.

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