

Comparison of *in vitro* and *in vivo* reference genes for internal standardization of real-time PCR data

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*Real-time PCR is a powerful technique for gene expression studies, which have become increasingly important in a large number of clinical and scientific fields. The significance of the obtained results strongly depends on the normalization of the data to compensate for differences between the samples. The most widely used approach is to use endogenous reference genes (housekeeping genes) as internal standards. This approach is controversially discussed in the literature because none of the reference genes is stably expressed throughout all biological samples. Therefore, candidate reference genes have to be validated for each experimental condition. In our studies, we introduced and evaluated an *in vitro* synthesized reference cRNA for internal standardization of relative messenger RNA (mRNA) expression patterns. This reference, consisting of the *in vitro* transcribed coding sequence of aequorin, a jellyfish protein, was incorporated in the extracted RNA. The experimental significance of this approach was representatively tested for the expression of the neurotrophin-3 mRNA in distinct regions of mouse brains. A comparison to three stably expressed reference genes [β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyl-transferase 1 (HPRT1)] gave evidence that the spiking of template RNA with *in vitro* transcribed cRNA is a valuable tool for internal standardization of real-time PCR experiments.*

INTRODUCTION

Real-time PCR is a sensitive and accurate technique to compare the messenger RNA (mRNA) expression of target genes in certain reverse-transcribed samples. A crucial step of this method is the normalization of the results to compensate for differences in the purity and concentration of the samples that were introduced during the sample preparation procedure. As in most analytical methods, these differences can be equalized by normalization to an internal standard. The most commonly used internal standards in real-time PCR are endogenous reference genes, also called housekeeping genes. A prerequisite for the use of these genes is that they are stably expressed at the same level throughout all samples (1) and that their expression is not influenced by the experimental conditions (e.g., drugs applied in the assay). Unfortunately, an ideal reference gene that complies with these requirements under different

experimental circumstances has not previously been described. The standard procedure is to determine a large set of putative reference genes for each experimental set individually (2). On the basis of these data, valid ones are selected. Vandesompele et al. (1) suggested a pairwise comparison of these genes and to assume that stably expressed genes show identical expression patterns throughout all samples. A less intricate principle is to normalize the results to the amount of total RNA. But in this case, the varying efficiencies of the reverse transcription or the PCR itself are not taken into account (3).

An alternative solution is to spike the total RNA with an *in vitro* transcribed RNA (cRNA), without homology to the total RNA sequences. Due to the fact that, in gene expression studies, using microarray techniques such cRNAs have proven to be a valuable tool for the normalization process (4), it is surprising that until now this principle

has not been validated for real-time PCR (3).

In the present study, we sought experimental proof for the suitability of this approach. We measured a representative target gene in different areas of mouse brain and compared the normalization of these results, based on various reference genes, with normalization using a cRNA added to the total RNA. For this internal standard, we chose the coding sequence of aequorin (GenBank® accession no. L29571), a jellyfish (*Aequorea victoria*) photoprotein, which exhibits no homology with the mouse genome. As reference genes, we selected β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyl-transferase 1 (HPRT1). To judge their validity as internal reference genes, we tested whether the choice of any of these internal standards affected the results of our target gene expression analysis. As a representative target gene, we measured the mRNA encoding the neurotrophic peptide, neurotrophin-3.

MATERIALS AND METHODS

Animals

For the experiments, we used four age-matched adult (3 months) male mice (C57BL/6J). The animals had free access to food and water and were kept at a constant room temperature (24°C), under a 12-h light/dark cycle (light on at 7 a.m.). Animals were maintained according to the guidelines of the European Union (Guideline 86/609/EWG).

RNA Preparation and Reverse Transcription

Brains from decapitated mice were rapidly removed, and brain regions [olfactory bulb, cerebellum, cortex, hypothalamus, hippocampus and brainstem (including thalamus and midbrain structures), and striatum] were rapidly dissected. RNA from brain sections was isolated using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), with DNase I treatment according to the

Table 1. Primer Sequences and PCR Efficiencies

Primer	Sequence	GenBank® Accession No.	Product Size (bp)	Efficiency (E ± SEM)
<i>GAPDH</i> ^a				
Sense	5'-TGCACCACCAACTGCTTAGC-3'	NM_008084	86	2.01 ± 0.01 n = 7
Antisense	5'-GGCATGGACTGTGGTCATGAG-3'			
<i>HPRT1</i> ^a				
Sense	5'-TGACACTGGCAAACAATGCA-3'	BC004686	94	2.03 ± 0.01 n = 11
Antisense	5'-GGTCCTTTCCACCAGCAAGCT-3'			
<i>β-Actin</i>				
Sense	5'-TCCATCATGAAGTGTGACGT-3'	NM_007393	112	1.97 ± 0.05 n = 11
Antisense	5'-GAGCAATGATCTTGATCTTCAT-3'			
<i>NT-3</i> ^b				
Sense	5'-CCGGTGGTAGCCAATAGAACC-3'	NM_008742	119	2.07 ± 0.02 n = 3
Antisense	5'-GCTGAGGACTGTGCGGTAC-3'			
<i>Aequorin</i>				
Sense	5'-TTGACGAGATGGTCTACAAGGCATC-3'	L29571	100	2.04 ± 0.05 n = 3
Antisense	5'-GAAGGCTTCTACAGCATCTTTGTGTCGT-3'			

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyl-transferase 1; NT-3, neurotrophin-3.
^aSequence of primers taken from RTPPrimerDB (17).
^bSequence of primers taken from Primerbank (18).

manufacturer's instructions. RNA was quantified spectrophotometrically (UVmini 1240; Shimadzu, Duisburg, Germany) at 260 nm. The RNA purity was confirmed as a 260/280 nm ratio above 1.8. The integrity of the RNA was verified by agarose gel electrophoresis. The absence of genomic DNA was initially checked in a real-time PCR containing 20 ng of total RNA (without reverse transcription) and *GAPDH*-specific primers (Table 1).

For synthesis of the cRNA, we used the coding sequence (600 bp) of aequorin, a jellyfish photoprotein. The sequence was originally derived from cytAEQ/pcDNA1 (Molecular Probes, Leiden, The Netherlands) and subcloned into *HindIII/XbaI*-digested pcDNA 3.1/Zeo (+) (Invitrogen, Karlsruhe, Germany). This sequence showed no significant homology to the mouse genome as determined by a BlastN search of the National Center for Biotechnology Information (NCBI) genome database. Initially, 1 µg of plasmid was digested using the restriction enzymes *XbaI* and *SmaI* (Fermentas, St. Leon-Rot, Germany) to isolate a DNA fragment containing the aequorin cDNA and the T7 promoter 5' from the aequorin start codon. A 600-bp cRNA was in vitro transcribed

according to the manufacturer's instructions (MAXIscript® T7 Kit; Ambion, Huntingdon, UK). The cRNA was DNase I treated (Fermentas) and purified (RNA Mini Kit; Qiagen) including a second DNase I treatment on the column. Prior to use, the cRNA was quantified spectrophotometrically and confirmed free of residual template DNA by real-time PCR. We performed a real-time PCR containing 10⁶ copies of the cRNA (not reverse transcribed) and aequorin-specific primers (Table 1).

The cRNA (320 fg corresponding to 10⁶ copies) was added to 1 µg of total RNA and reverse transcribed in a volume of 20 µL according to the manufacturer's instructions using random hexamer primers (RevertAid™ First Strand cDNA Synthesis Kit; Fermentas). For quantitative real-time PCR, 35 µL amplification mixtures (QuantiTect® SYBR® Green Kit; Qiagen) were prepared containing cDNA (equivalent to 20 ng reverse-transcribed RNA), 300 nM primers (Table 1), and 10 nM fluorescein (Bio-Rad Laboratories, Munich, Germany), according to the manufacturer's instructions. Reactions (triplicates; 10 µL) were run on an iCycler iQ® Real-Time PCR Detection System (Bio-Rad

Laboratories). The cycling conditions were 15 min polymerase activation at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. The results were analyzed using the iCycler iQ Optical System Software Version 3.0a (Bio-Rad Laboratories). The baseline was manually set and the threshold automatically set by the software. The crossing point of the amplification curve with the threshold represents the cycle threshold (C_t). Results were exported to Microsoft® Excel® for further analysis.

In the present study, we compared all tissues with the largest assessed being the cortex. The relative mRNA expression

was calculated from the ratio of sample and control (cortex) according to Pfaffl (5) (see Equations 1–3). The relative quantity was determined using the mean calculated efficiency (E) (Equation 1). This parameter was derived for each pair of primers from standard curves (5 points in triplicates; cDNA corresponding to 50 ng to 5 pg reverse-transcribed RNA of cortex cDNA). The results for the three reference genes (*β-actin*, *GAPDH*, and *HPRT1*) and the cRNA were determined by the same method. In the next step, each target result was normalized separately with respect to the reference genes (Equation 3), and the resulting values were displayed as log₂ values.

$$E = 10^{-1/\text{slope}} \quad [\text{Eq. 1}]$$

$$R = E^{(C_{t \text{ control}} - C_{t \text{ sample}})} \quad [\text{Eq. 2}]$$

$$\text{Ratio} = R_{\text{target}}/R_{\text{reference}} \quad [\text{Eq. 3}]$$

The size of the PCR products was initially analyzed by agarose gel electrophoresis, and the identity of the targets was confirmed by dideoxy chain termination sequencing. In addition, the real-time reaction of the products was checked by melting point analysis after each reaction.

Data Analysis

For the calculation of the results, we developed qCalculator 1.0, a Visual Basic for Applications (VBA)-based analysis tool for Excel. It can be downloaded from our homepage (www.pharmakologie.uni-bonn.de/frames/index_fr.htm). It enables a flexible calculation of 32 samples (in triplicates) for 20 experiments based on up to 10 reference genes including an automatic statistical comparison of different choices of reference genes.

Shown are arithmetic means \pm SEM of n experiments. The two-tailed Student's t -test was used to compare results under the precondition that their SEM values did not differ significantly. The f -test was used to verify this requirement.

GraphPad Prism[®] 4.0 (GraphPad Software, San Diego, USA) was used to test the normal distribution of the results using the Kolmogorov-Smirnov

test (KS-test) and to calculate the associated P values according to Dallal and Wilkinson (6).

RESULTS AND DISCUSSION

The integrity of the RNA is essential for the validity of the quantification of RNA expression profiles. A recent publication demonstrates (7) that degradation differentially alters the stability of reference genes. Therefore, in this study, only intact RNA samples were assessed. In addition, high-quality RNA was essential to ensure a valid quantification of the RNA concentrations of the samples.

All PCR products exhibited melting curves indicating the amplification of a single product (data not shown), and sequencing data from the products perfectly matched the corresponding gene in the NCBI database (Table 1).

Standard curves of all genes examined showed high efficiency over a 10^5 -fold range of cDNA concentrations (Table 1).

To evaluate the usefulness of the cRNA, we analyzed different amounts (10^4 – 10^8 copies) of the cRNA. Because the efficiency of the reverse transcription reaction depends on the total amount of RNA (8), we added constant amounts (1 μ g) of total RNA to each dilution. After reverse transcription, the resulting cDNA was quantified. A representative experiment (Figure 1) shows a highly significant linear correlation between the amount of cRNA and the corresponding C_t value. The mean efficiency of three experiments (2.03 ± 0.05) was not significantly different from that obtained from cDNA dilution curves (see Table 1, aequorin). Thus, it made no difference whether the incorporated cRNA or the resulting cDNA was diluted. This indicates that the expression patterns of the RNAs were identical to those in the reverse-transcribed cDNA. Based on these results, we decided to add 10^6 copies of cRNA per microgram of the total RNA to obtain C_t values in the same order of magnitude as those for the neurotrophin-3 (NT-3; data not shown). Furthermore, total RNA was spiked with cRNA (10^6 copies per microgram), and this mixture was serially diluted (2-fold; 4.0–0.1 μ g) and reverse transcribed. The real-time PCR results (Figure 2) of the cRNA and the representative target gene *GAPDH* show linearity in a parallel mode over the assessed range. The mean efficiencies did not differ significantly, indicating identical reverse transcription and PCR efficiencies (*GAPDH*: 2.2 ± 0.3 ; cRNA 2.1 ± 0.1 , $n = 4$).

In general, analyzing ratios as derived from Equation 3 can lead to problems because ratios are intrinsically asymmetrically distributed (9). Thus, higher expressed genes are expressed as values greater than one whereas lower expressed genes produce values between zero and one. One solution is to use geometrical means as presented by Vandesompele et al. (1). Another possibility is to transform the ratios into logarithmic values. Then the range of values increases from zero to infinity and decreases from zero to

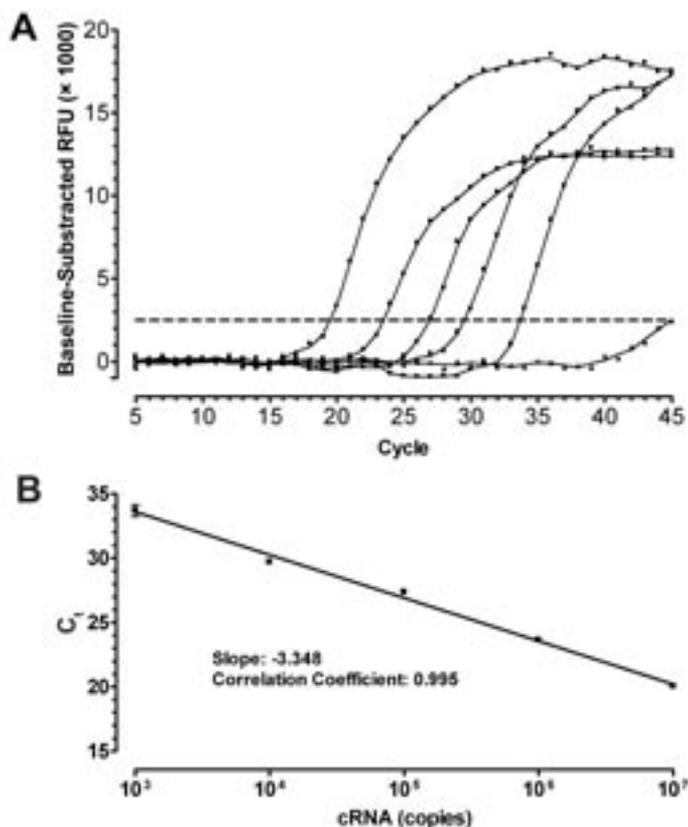


Figure 1. Representative results of a 10-fold serial dilution of cRNA added to a constant amount of total RNA. Serially diluted cRNA (5×10^4 to 5×10^8 copies) was added to 1 μ g of cortex total RNA and reverse transcribed. Each reaction contained 20 ng (RNA equivalent) of the resulting cDNA. (A) Shown are representative PCR curves of the reverse-transcribed cRNA (aequorin, square symbols) and a negative control (triangular symbols) and (B) a linear regression of the results (triplicates). RFU, relative fluorescence unit.

negative infinity. It should be remarked that both approaches lead to mathematically identical results because the geometric mean is the antilog of the mean of logarithmic values (9). The advantage of these logarithmic values is that they are theoretically normal distributed, which is a precondition for the calculation of arithmetic mean values and further statistical analysis (e.g., *t*-test, *f*-test). To prove these theoretical considerations, we assessed representative results for β -actin (data not shown). They provided evidence that ratios derived from Equation 3 were not normal distributed ($P < 0.0001$; $n = 201$) while the \log_2 ratios were normal distributed ($P > 0.05$; $n = 201$). Based on this result, we chose these logarithmic values for our calculations. This result is in good agreement with findings of Livak and Schmittgen (10) who propagated a similar distribution for $-\Delta\Delta C_t$ values, which have a comparable dimension, and with the recommended analysis of data derived from microarray experiments (11,12).

In our experiments, the mRNA encoding NT-3 is differentially expressed throughout the mouse brain. Compared with the cortex, the largest brain region assessed, the mRNA expression level was approximately 4-fold higher in the hippocampus and cerebellum and 16-fold lower in the olfactory bulb (Figure 3).

The results obtained from all investigated brain regions were not affected by the choice of the reference gene or the cRNA (Figure 3). Even the error levels of the results do not differ significantly. These results suggest that each of the assessed reference genes enables proper normalization. They also show that the in vitro synthesized cRNA used in this study is an alternative to reference genes as internal standards for real-time PCR. In this study, it was preferable to add the cRNA in a fixed relation to the quantity of total RNA, as opposed to a calculation based on the amount of the biological material as shown by Smith et al. (13), due to the fact that different tissues express varying amounts of total RNA. In addition, we know from our own experience that extraction efficiencies depend on the total amount of starting material and tend to vary from sample to sample. Beside these

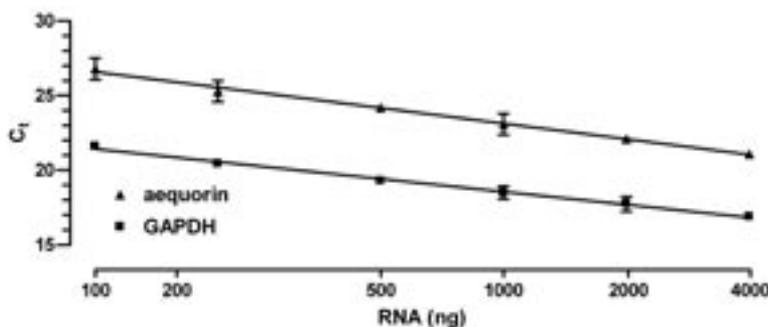


Figure 2. Representative experiment of a dilution series of RNA spiked with an in vitro transcribed cRNA. Four micrograms of total RNA were spiked with cRNA (aequorin, 1×10^6 copies/microgram of RNA), diluted, and reverse transcribed. The resulting cDNAs of GAPDH (squares) and aequorin (triangles) were quantified. Each reaction contained 2% (0.4 μ L) of the reverse transcription reaction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

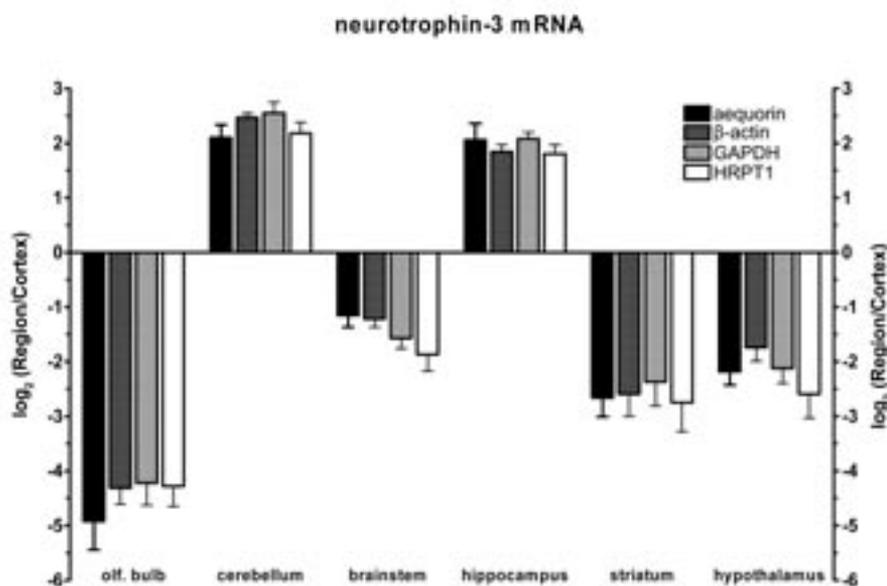


Figure 3. Influence of in vivo and in vitro reference genes on the relative expression of mRNAs encoding NT-3 in mice brain. Shown are the relative expression values of various brain regions compared with the cortex. Tissue RNA (1 μ g) was spiked with 10^6 copies of cRNA (aequorin) and reverse transcribed. Neurotrophin-3, cRNA, and endogenous reference genes (β -actin, GAPDH, and *HRPT1*) were measured by real-time PCR. Shown are results from four independent experiments. mRNA, messenger RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *HRPT1*, hypoxanthine phosphoribosyl-transferase 1; olf. bulb, olfactory bulb.

drawbacks, the mentioned calculation based on the amount of starting material is preferable if a valid quantification of the RNA is not possible (e.g., single cells or microdissected tissues). The assessment of RNA quantities measures predominately the ribosomal RNA fraction. When working with tissues, where an imbalance between messenger and ribosomal RNA is suspected (14,15), we suggest using an additional reference gene to identify this systematic error. An advantage of the cRNA chosen in the present study is that the sequence is unique

for *A. victoria* and related species and, consequently, can also be used in many other research fields. It should be mentioned that the presented cRNA is not polyadenylated, therefore oligo(dT) primers are not suitable for the reverse transcription. If desired, a poly(A) tail may be introduced into the template cDNA by genetic engineering.

While the results discussed here show that the reference genes in our study were stably expressed in different areas of the mouse brain, it is known from the literature (3) that this is not always the case, particu-

larly for *GAPDH* and β -*actin* under certain experimental conditions. They are therefore not always appropriate as reference genes (16). The large number of publications that focus on the validation of internal reference genes shows the difficulty in selecting suitable candidates. In the case of experiments using a limited number of samples, the effort expended to obtain a valid internal standardization based on reference genes is often greater than that for the main research work, emphasizing the value and advantage of using introduced cRNA for normalization. In comparison to standard reference genes, in vitro transcribed reference RNA is a more universal, alternative approach that does not require validation for each experimental setting.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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