Endothelial dysfunction in experimental models of preclinical diabetic retinopathy

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Chapter 4

Normalization of quantitative PCR in complex diseased tissues

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Abstract

Real-time quantitative PCR (qPCR) is the method of choice for quantification of specific mRNA transcript levels in tissues. The interpretation of qPCR data relies heavily on normalization, which should correct for sampling errors, qPCR efficiency variations and other sources of variation. Normalization algorithms relying on expression levels of a single “housekeeping” gene or a set of genes are often unreliable, as the expression of these genes may also be altered as a result of the experimental conditions under investigation. We introduce here a normalization procedure that is based on the combination of wet weight of tissue, total amount of RNA and the total amount of synthesized first strand complementary DNA (cDNA). The method is rapid, cheap and can be used under all conditions including complex diseases and can be applied to tissues consisting of different cell types.
Introduction

Real-time qPCR has become the method of choice for quantifying mRNA transcript levels in biological samples because of its high sensitivity, wide dynamic range, accuracy and suitability for high-throughput screening.\textsuperscript{1,2} qPCR is particularly useful for studying the many, often subtle, changes in gene expression in diseased tissues. However, its sensitivity makes qPCR prone to inaccuracies and errors based on variation in sample handling, extraction protocols, co-purification of inhibitors of reverse transcriptase (RT), efficiency of the RT reaction,\textsuperscript{3} PCR efficiency,\textsuperscript{4} and many other factors as summarized in Fig. 1. Therefore, it is common practice to employ a normalization procedure to correct for sample-to-sample variability, assuming that all transcripts in a sample are affected to the same degree.\textsuperscript{4} Various methods for normalization of qPCR data have been developed. However, a standard normalization procedure for qPCR studies is not yet available.

Such a standard normalization procedure should produce a normalization factor that 1) shows minimal variance across a sample set and should 2) not show statistically significant differences between different treatment groups.

We developed a novel normalization approach based on the combination of wet weight of tissue, total amount of RNA and total amount of synthetized cDNA (Box 1). We compared the effects of different normalization methods on transcription levels of the Icam-1 gene that is distinctly upregulated at the mRNA and protein level in diabetic retina.\textsuperscript{14-16}

Methods of normalization

The first obvious step in normalization is the use of similar sample sizes on the basis of tissue volume, weight or cell count.\textsuperscript{4} This step is often hampered by the heterogeneity of cell types in a tissue or differences in tissue morphology due to disease or experimental conditions.

Normalization can also be achieved on the basis of identical total amounts of RNA in all samples. Total RNA can be quantified spectrophotometrically at 260 nm, but this is not a very accurate measure for the mRNA content of a sample. First, total RNA consists for over 80% of rRNA and any change in the rRNA:mRNA ratio is not taken into account.\textsuperscript{5,6} Second, the method does not correct for traces of genomic DNA, that are also measured at 260 nm and interfere with amplification efficiency.\textsuperscript{7,8} Furthermore, variations in the RT or PCR reactions are not corrected for in this normalization approach.

The most commonly used method for normalization is based on the expression of a single internal reference gene often referred to as a “housekeeping” gene. It is assumed that this gene is constitutively expressed and that its transcript levels are not altered by disease or experimental conditions. Frequently used reference genes are glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin, 18S rRNA and 28S rRNA.\textsuperscript{9}
Adult Wistar rats (Charles River, Maastricht, The Netherlands) were randomly divided into two experimental groups: a control group (n=14) and a diabetic group (n=16). Diabetes was induced by streptozotocin as described previously. After 6 weeks, half of the rats from each group were killed and after 12 weeks the other half. Wet weight of the retinas was determined and total RNA was extracted from retinas with TRIzol reagent (Invitrogen, Carlsbad CA, USA). The amount of total retinal RNA isolated was 14 ± 3 μg per retina (mean ± SD, spectrophotometric measurements at 260 nm), with no significant differences between the experimental groups. The integrity of the RNA samples was verified using an automated electrophoresis system (Experion; Bio-Rad, Hercules CA, USA). All samples had sharp ribosomal RNA bands with no sign of degradation. A 2.0 μg aliquot of total RNA was treated for 15 min with 0.5 U DNase I (Invitrogen) and reverse transcribed into first strand cDNA with Superscript II and oligo(dT)12-18 (Invitrogen), yielding a 20 μl cDNA sample.

Real-time qPCR was performed in a sequence detection system (ABI Prism 7300; Applied Biosystems, Foster City CA, USA). For each primer set, a 20 μl/sample mastermix was prepared consisting of 1× SYBR Green PCR buffer (Eurogentec, Seraing, Belgium), 3 mmol/l MgCl2 200 μmol/l each of dATP, dGTP and dCTP, 400 μmol/l dUTP, 0.5 U AmpliTaq Gold (Eurogentec) and 0.1 μmol/l primers. All cDNA samples were diluted 1:10 and 1 μl was amplified using the following PCR protocol: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and a dissociation program (60–95°C). Details of the primers are given in Table 1. Whenever possible, the forward and reverse primers were placed in separate exons. Specificity of the primers was confirmed by BLAST searching. The presence of a single PCR product was verified by the presence of a single peak in the dissociation profile and the detection of a single band of the expected amplicon size on a 3% agarose gel. Relative gene expression was calculated using the equation: R=E−Ct, where E is the mean amplification efficiency of all samples for the gene being evaluated and Ct is the cycle threshold for the gene as determined during real-time PCR. All qPCR experiments were performed at least twice.

Quantification of the amount cDNA synthesized during the RT reaction was performed according to Sambrook et al. [α-32P]-dCTP was incorporated in the cDNA during the RT reaction. For this purpose, a ‘hot-mix’ was prepared of dNTPs containing 2.5 mmol/l each of dATP, dTTP, dGTP, dCTP and 1.67 μmol/l [α-32P]-dCTP (MP Biomedicals, Amsterdam, The Netherlands). Validation of the method was performed with a series of 2-fold serial dilutions of pooled total RNA that was reverse transcribed using the ‘hot-mix’. Two μl cDNA with [α-32P]-dCTP incorporated was transferred from each sample onto separate DE81 filters (Whatman, ’s-Hertogenbosch, The Netherlands) and allowed to dry. To remove all non-incorporated [α-32P]-dCTP nucleotides, filters were thoroughly washed three times in 0.5 mol/l phosphate buffer (pH 7.0) followed by one wash in ice-cold 70% ethanol. The amount of radioactivity bound to the filters was measured as counts per minute (cpm) using a scintillation counter (Beckman Coulter, Mijdrecht, The Netherlands). The relation between cpm values representing cDNA and known amounts of total RNA in a series of dilutions was linear (Fig. 2). Moreover, the relationships between wet weight of retinas and total amount of RNA and between total amount of RNA and the amount of cDNA of all retinas included in the study (n = 30) were linear as well (Fig. 3).

However, expression of these genes is not as stable in varying cell types and under different experimental conditions, as once was assumed, rendering this normalization method doubtful. This is demonstrated in Box 2 and Fig. 4 for a number of these genes in rat control and diabetic retinas.

Another frequently applied normalization method is the Excel-based platform geNorm, which assesses the correlation of transcript levels of multiple reference genes.
Figure 1. Considerations for real-time RT-qPCR. Many factors in the steps towards the production of cDNA may affect the outcome of the qPCR analysis. Therefore, normalization on the basis of sample size or total RNA may be biased. Furthermore, amplification of specific PCR products is dependent on the secondary structure of the cDNA and the primer efficiency and specificity. Careful selection and validation is necessary when normalization is performed using reference genes. Normalization on the basis of cDNA lacks the disadvantages of other normalization methods.

in all samples by pairwise comparison. A set of two or more genes with the lowest pairwise variation are selected and subsequently used to calculate a sample-specific normalization factor from the geometric mean of the transcript levels. The reasoning behind this approach is the assumption that genes which are stably-expressed under experimental conditions show a similar pattern in fluctuation in expression as in the control samples, whereas genes that are truly up- or downregulated under experimental conditions display a unique fluctuation pattern of expression. This leads to a higher pairwise variation compared to the more stably-expressed genes. However, this method is problematic when co-regulated genes are among the candidate reference genes, since the method does not check for variation between experimental groups. This can lead to a significant data bias. A model-based method for estimation of variation in expression, Normfinder, does take into account the variation between subgroups of the sample set and is less sensitive for co-regulation. However, this method still has the disadvantage that multiple candidate reference genes have to be validated to measure
Table 1. Gene nomenclature, GenBank accession code, primer sequences, predicted size (bp) and melting temperature (Tm) of the amplified product for rat genes studied with real-time quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
<th>bp</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icam-1</td>
<td>NM_012967</td>
<td>CTGGTCCCTCCAATGGCTTCAAC</td>
<td>TGTGGGATGGATGGATACCTGAG</td>
<td>113</td>
<td>86</td>
</tr>
<tr>
<td>Actb</td>
<td>NM_031144</td>
<td>AGCCATGTACGTAGCCATCCA</td>
<td>TCTCCGAGTCACCATCAATG</td>
<td>81</td>
<td>84</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_017008</td>
<td>TCACCACATGGAGAAGGG</td>
<td>GCTAACGATGGGTGGGCA</td>
<td>169</td>
<td>87</td>
</tr>
<tr>
<td>Hmbs</td>
<td>NM_013168</td>
<td>ATGGGCAACTGTACCTGACTGG</td>
<td>CCATCTTCTTGTGACACAGGA</td>
<td>106</td>
<td>81</td>
</tr>
<tr>
<td>Nef3</td>
<td>NM_017029</td>
<td>TCACCAGATAGAACTACTG</td>
<td>TAGGCCGTCCGGTGTGTCG</td>
<td>97</td>
<td>82</td>
</tr>
<tr>
<td>Pde6b</td>
<td>XM_214126</td>
<td>GTTGCGTTCATCGACTTGTATG</td>
<td>GGTAACATGGGCGAGTCT</td>
<td>82</td>
<td>78</td>
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<tr>
<td>Prkca</td>
<td>XM_343975</td>
<td>TCAATTGGGCTTCCGGAT</td>
<td>TGGATCGGATGGTTTTGTT</td>
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<td>79</td>
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<tr>
<td>Rho</td>
<td>NM_033441</td>
<td>GCAACAGGATGCGCTACCA</td>
<td>GCATAGGGAAAGCAGCACAT</td>
<td>99</td>
<td>82</td>
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<tr>
<td>Tbp</td>
<td>NM_001004198</td>
<td>ACCAGAAACACGCTCTCCACCTT</td>
<td>TGGAGTACGCGTGGCCTACGTA</td>
<td>116</td>
<td>82</td>
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<tr>
<td>Tuba2</td>
<td>NM_00104276</td>
<td>CATGCCCAGGTTGACAATTCA</td>
<td>AGGCAAGGCTTCGGCATAATCA</td>
<td>167</td>
<td>84</td>
</tr>
<tr>
<td>Ubc</td>
<td>NM_017314</td>
<td>TCGTGACCTCTCCACGAGTATCTGA</td>
<td>GAAACTAAAGACACCTCCCATCA</td>
<td>82</td>
<td>79</td>
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<tr>
<td>Ywhaz</td>
<td>NM_013011</td>
<td>CAAGCATAACAGAAAGCATTGA</td>
<td>GGGCGACAGCAGTCTGA</td>
<td>76</td>
<td>78</td>
</tr>
</tbody>
</table>

their estimated expression variation.

On a theoretical basis, we predicted that total cDNA is the best option for normalizing real-time RT-qPCR data, because the amount of generated cDNA is only one step away from both the mRNA and the PCR product. The cDNA normalization approach corrects for variations in sample size, RNA levels, rRNA:mRNA ratio’s, efficiency of RT reactions and avoids the labor-intensive task of finding valid reference genes for normalization (Fig. 1). However, when total cDNA is taken for normalization, experimental errors are not taken into consideration. Therefore, extra controls are included by relating the amount of cDNA to total amount of RNA and wet weight of retina (Fig. 3). The linear relationships observed between these three measurements are indicative of a well executed experiment with minimal sample handling error. On this basis it is concluded that cDNA is a good normalization parameter in this study. Expression levels of the Icam-1 gene were normalized on the basis of the different normalization procedures in Box 3 and Fig. 5. It is shown that the outcome of the various normalization procedures varies. Similar expected results were obtained when wet weight of retina, total amount of RNA, cDNA and expression of the genes Tuba2 and Rho were used.
To test the robustness of the cDNA normalization method, we used a larger set of samples (n=33) and analyzed the effect on sample variance of 48 transcripts by the different normalization methods described above (Fig. 6). This study contained data of retinas from two additional groups of animals, either with STZ-induced diabetes or after aminoguanidine treatment, as described elsewhere. Compared to normalization on the basis of total RNA (non-normalized), normalization on the basis of total cDNA showed a lower average coefficient of variation (CV) of the 48 transcripts (Fig. 6A). The lower CV of cDNA-normalized as compared to non-normalized CV values was not significant, as determined by a paired t-test. CV values obtained by normalization on the basis of wet weight of retina resulted in significant higher CV values. Only normalization with geNorm resulted in a significantly lower average CV, but showed a larger spread of variation. However, the current analysis does not take into account that some
transcripts are strongly regulated by the treatment conditions. Therefore, we also analyzed the CV of the control samples (n = 6) as a separate data set (Fig. 6B). Compared to the complete sample set, the average CV derived from the different normalization methods showed lower values in the controls, suggesting that the treatment conditions contribute to the variance. Normalization on the basis of Gapdh and Rho expression gave the poorest performance in terms of average CV in the complete sample set but performed much better in the ‘controls-only’ sample set. This suggests that the variation in the treatment groups is large. Normalization by geNorm and Normfinder resulted in a similar average CV, which was lower than that of normalization on the basis of cDNA. However, these two normalization methods cannot be applied because of their statistically significant differences between treatment groups. Finally, gain of performance was obtained in the ‘controls-only’ sample set when data were normalized with the cDNA method as compared to the total RNA method. This is shown by a

**Figure 4.** Expression of candidate reference genes in rat retina after induction of diabetes using total cDNA for normalization. Expression of candidate reference genes was significantly increased at 6 weeks (grey bars) and/or 12 weeks (black bars) of STZ induced diabetes in comparison to healthy retinas (white bars; set at 1) with the exception of Tuba2 and Rho levels. Means and standard deviations are given. Significant differences (p < 0.05) between expression levels in diabetic retinas and healthy retinas are indicated by an asterisk.

**BOX 2. Reference genes for normalization**

RT samples of control and diabetic rat retina with and without ‘hot-mix’ were made in triplicate. Variation in the resulting cpm values was 20%. Real-time qPCR was performed with primers (Table 1) for eleven candidate reference genes (seven frequently-used reference genes: Actb, Gapdh, Hmbs, Tbp, Tuba2, Ubc, Ywhaz and four abundantly-expressed retina-specific genes: Nef3, Pde6b, Pkca and Rho) and the Icam-1 gene.

To show the effect of diabetes on qPCR data of the eleven reference genes, their expression levels were normalized on the basis of cpm values for each sample, representing the amount of cDNA (Fig. 4). Normalization on the basis of wet weight of retina or total amount of RNA gave similar results (data not shown). Except for Tuba2 and Rho, all candidate reference genes showed statistically significant alterations (single ANOVA) in expression levels in the diabetes retinas as compared to healthy retinas. This demonstrates that most reference genes are not stably expressed under diabetic conditions, as was shown previously.19-22
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significant decrease of CV values (Fig. 6B), confirming the added value of normalization on the basis of cDNA.

We found that normalization on the basis of cDNA showed the lowest average CV in a large data set without statistically significant differences between treatment groups. Furthermore, the method is cheap, quick, easy to perform and sensitive. We present here a method with incorporated [\(\alpha^{32}\text{P}\)]-dCTP nucleotides. A recently published method using the incorporation of ribogreen after reverse transcription corroborates our findings. To check for experimental errors, normalization on the basis of wet weight of tissue and of total RNA has to be included in the procedure.

Our results confirm reports by other authors that “housekeeping” genes are not independent of the experimental conditions tested, especially not in complex tissues such as the retina or in complex diseases such as diabetes. For example, \textit{Gapdh} has previously been shown to be upregulated in an animal model of diabetes and in vitro under high glucose conditions as well as in other conditions (insulin treatment, oxidative stress, hypoxia).

\textit{Gapdh}, \textit{Actb} and \textit{Ywhaz} have been reported by the geNorm application to be the most stable reference genes in various cell types and neuroblastoma. Also in our study the combination of these three genes came up as the most stably-expressed set of candidate reference genes. In our diabetes data set, the geNorm method successfully reduced CV. However, when performing statistical analysis (single ANOVA) with these three genes and the normalization factor resulting from that, significant differences between

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{The effect of different normalization methods on \textit{Icam-1} gene expression data in rat retina. \textit{Icam-1} expression levels in control retina (white bars, set at 1) and retinas after 6 weeks (grey bars) and 12 weeks (black bars) of STZ induced diabetes were normalized on the basis of total retina, total RNA, total cDNA, expression levels of two frequently used housekeeping genes (\textit{Gapdh} and \textit{\(\beta\)-actin}), the expression levels of two reference genes that showed no variance between the three experimental groups (\textit{Tuba2} and \textit{Rho}), the geometric mean of three most stably-expressed genes as determined by geNorm, or by the geometric mean of two most stably-expressed genes as determined by Normfinder. Means and standard deviations are given. Significant differences (\(p < 0.05\)) between expression levels in diabetic retinas and healthy retinas are indicated by an asterisk.}
\end{figure}
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BOX 3. Normalization of ICAM-1 gene expression

The effect on the expression levels of *ICAM-1* by normalization with total cDNA was compared with normalization by other methods (Fig. 5). Again, when wet weight of tissue or total amount of RNA was used for normalization, similar expression patterns were found. The following conclusions can be drawn on the basis of this comparison:

(i) When the expression data were normalized on the basis of cDNA, *ICAM-1* expression was significantly upregulated after 6 and 12 weeks of diabetes (1.8 and 2.7-fold, respectively). (ii) *ICAM-1* expression normalized on the basis of total retina resulted in a not significant upregulation of 1.6 and 2.2-fold after 6 and 12 weeks of diabetes, respectively. (iii) Normalization based on total AMOUNT OF RNA resulted in significantly upregulated *ICAM-1* levels after 6 and 12 weeks of diabetes (1.8 and 2.5-fold, respectively), which was similar to normalization based on total cDNA. (iv-v) When two frequently-used reference genes, *Gapdh* or *β-actin*, were used for normalization no increase or only a small increase in *ICAM-1* expression was found in diabetic retinas. The expression levels of these genes were statistically different from each other in the control and diabetes groups.

(vi-vii) *Tuba2* and *Rho* were the only two genes that showed no statistical difference in expression levels between control and diabetes groups before and after its use as a reference gene. *Rho* also showed the lowest estimated intra- and intergroup variation based on the Normfinder program. Normalization on the basis of *Tuba2* and *Rho* expression levels showed similar patterns as compared to normalization based on cDNA, but resulted in increased variance. (viii) The combined use of *Ywhaz*, *β-actin* and *Gapdh* expression appeared to be the most stable when the geNorm program was used for normalization. When *ICAM-1* expression was normalized with the geometric mean of the expression data of these genes, *ICAM-1* expression was similar in control and diabetic retinas. This was caused by the fact that expression data of these genes (*Ywhaz*, *β-actin* and *Gapdh*) showed statistically significant differences between control and diabetes groups before and after normalization indicating co-regulation of these reference genes. (ix) The Normfinder application ranked the combination of *Gapdh* and *Tbp* as the most stable combination of genes. Although this program is designed to eliminate the effect of co-regulated genes, statistically significant differences were found between control and treatment groups in the data sets of *Gapdh* and *Tbp*, before and after normalization, and the normalization factor derived from it.

groups were found, a disadvantage of this application also mentioned by others. In fact, Vandesompele et al. advise against the use of co-regulated genes, but do not advise how to avoid this pitfall. Moreover, co-regulation can not always be predicted, as was demonstrated by Andersen et al. In the present study, it is shown that for qPCR normalization a valid and reliable reference gene, or a combination of reference genes, can be difficult to identify, especially in complex tissues and diseases.

Incorporation of radiolabeled deoxyribonucleotides into the first strand cDNA is linearly related to the amount of template mRNA. We used total RNA of high quality without signs of degradation and we used oligo(dT) primers in the RT reaction, which are specific for poly-A tails of intact mRNA transcripts. Therefore, it is safe to assume that the amount of cDNA in each sample is directly proportional to the amount of mRNA. This was also reflected by our extra control normalization steps, since normalization on the basis of wet weight of tissue, total RNA and cDNA gave similar results. In fact, this finding argues against normalization. Rather total RNA or cDNA should be used for comparison of qPCR data of experimental groups. Total RNA and even
wet weight of tissue may be sufficient in many cases, but normalization on the basis of cDNA is an extra control for variation.

In conclusion, on theoretical grounds we predicted that using total sample cDNA content results in superior normalization of gene expression data when used in combination proper sample handling techniques. This was confirmed empirically in an animal model of diabetes.

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