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# Standardization strategy for quantitative PCR in human seminoma and normal testis

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**Abstract**

Housekeeping genes are commonly used as endogenous references in quantitative RT-PCR. Ideally these genes are constitutionally expressed by all cell types and do not vary under experimental conditions. Tissues of 9 normal testes and 22 classical pure seminoma were obtained for RNA-extraction. Real-time RT-PCR was used to examine the mRNA-expression of ubiquitin C, beta-actin, GAPDH, 18S ribosomal RNA (18S rRNA) and porphobilinogen-deaminase (PBGD). Additionally, 3 normal testicular tissues and 39 seminoma, including 1 normal testis and 17 seminoma of the RT-PCR group, were utilized for microarray analyses. Ubiquitin C (protein degradation) was down-regulated, GAPDH (carbohydrate metabolism), beta-actin (cytoskeleton), 18S rRNA (ribosome) and PBGD (porphyrin metabolism) were up-regulated in seminoma. A normalization of the target gene data with up-regulated housekeeping genes would equalize or underestimate up-regulated data and overestimate down-regulated data. We demonstrate that none of the investigated housekeeping genes is suitable for normalization of the target gene RT-PCR data, but may be essential for tumor metabolism in human seminoma. Further, we developed a standardization strategy, which is applicable to many experimental investigations.


*Keywords:* Beta-actin; GAPDH; Housekeeping gene; Quantitative PCR; Seminoma; Testis

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## 1. Introduction

There are different quantification strategies in real-time RT-PCR, such as absolute and relative quantification. The current method for relative quantification is to normalize the PCR-data by a reference gene for eliminating unspecific variation caused by differences in sample preparation, RNA extraction- or RT-efficiencies. The normalization by a so-called housekeeping gene is generally accepted, although evidence is growing, that the choice of a suitable housekeeping gene and the calculation of the normalized data may be much more complex than it seems to be (Vandesompele et al., 2002). Housekeeping genes maintain cellular function, are expressed at a constant level and are unaffected by experimental conditions. Used as a reference gene, they should further be expressed at a similar level as the target genes (Bustin, 2000). The most commonly used housekeeping genes are glyceraldehydes-3-phosphate-dehydrogenase (GAPDH), beta-actin, 18S rRNA and 28S rRNA (Suzuki et al., 2000). There are many references that document variegated circumstances, where housekeeping genes behave contrary to their predicted constant expression, e.g. GAPDH, an enzyme of glycolysis and gluconeogenesis, is regulated in different stages of the cell cycle, development, pregnancy and several cancers, and is influenced by dexamethasone, glucose, insulin, growth hormone, oxidative stress, hypoxia, apoptosis, tumor proliferation, starvation, UV-light, interleukin-2, norepinephrin and others (Bustin, 2000; Suzuki et al., 2000). Beta-actin is a cell-structure element and may be influenced by hypoxia, ionizing radiation, vitamin B6 deficiency, different growth factors, adenocorticotropin and gonadotropins (Bustin, 2000; Suzuki et al., 2000). Ubiquitin is involved in protein degradation and is up-regulated in 2,6-dichloro-4-nitrophenol (DCNP) or prostaglandin F 2 alpha (PGF2alpha) induced apoptosis (Qi and Sit, 2000; Young et al., 1998). While Fink et al. (1990) propagate porphobilinogen deaminase (PBGD, equivalent to hydroxymethylbilane synthase, HMBS) as a housekeeping gene for quantitative PCR, Lupberger et al. (2002) show that PBGD is differentially expressed in their experimental design with a lymphocyte cell line and leucocytes of normal individuals and of malignoma patients. They regarded beta2-microglobulin as the most suitable reference gene. There are marked expression differences between different sorts of cells and tissues (Warrington et al., 2000), so that suitable housekeeping genes have to be established for every new experimental design.

The purpose of our study was to find a standardization strategy for our real-time RT-PCR data acquired in human seminoma (a common tumor of the testis) and normal testicular tissue and to discuss potential consequences and risks of conventional normalization using housekeeping genes.

## 2. Materials and methods

### 2.1. Tissue collection

For quantitative RT-PCR investigations normal testicular tissue ($n = 9$) and seminoma tissue ($n = 22$) were collected, snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. One normal testicular tissue and 17 seminoma were additionally analyzed by microarray as described below. All patients underwent surgery between 1998 and 2002 at the Department of Urology, University Hospital Mannheim, Germany. Patients' informed consent was taken prior to all investigations. Histological diagnosis and evaluation of the tumor stage were performed by conventional light microscopy. Eight pT1- and 14 pT2-stages were identified. Whereas pT1-stages of seminoma are limited to the testis and epididymal tissues, pT2-stages are characterized by angio- and lymphangio-invasion or by infiltration of the tunica vaginalis.

For microarray investigations, 41 testicular tissue samples were analyzed, including 3 normal tissue samples and 39 pure seminoma. All patients underwent surgery between 1995 and 2002 at the Departments of Urology in Mannheim, Essen or Münster. Patients' informed consent was taken prior to all investigations. The distribution of seminoma stages was 22 and 11 cases for stage pT1 and pT2, respectively.

Histological characterization and purity of all samples were verified by frozen sections before RNA extraction.

### 2.2. Quantitative RT-PCR

Total RNA from 30 mg of normal testicular tissue and seminoma was extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) following

the animal tissue protocol of the manufacturer's instructions. Tissue lysates were homogenized with the QIAshredder (QIAGEN). RNA was dissolved in water and spectroscopically quantified at 260 nm with the Ultrospec 3100 Pro (Biochrom Ltd., Cambridge, UK). RNA was measured at neutral pH in 10 mM Tris–HCl and absorbance of the measured samples was between 0.15 and 1.0. The purity of RNA was verified by optical density (OD) absorption ratio $OD_{260\,nm}/OD_{280\,nm}$ between 1.80 and 2.06 (mean = 2.0). RNA quality was analyzed using the RNA 6000 Nano LabChip® Kit (Agilent Technologies GmbH, Böblingen, Germany) and the Agilent 2100 bioanalyser (Agilent Technologies) for electrophoretic separation. The 28S/18S rRNA ratio of all samples was between 1.7 and 2.0 (mean = 1.84). RNA quantities acquired by the Agilent 2100 bioanalyser were relatively regarded the same as measured spectrophotometrically.

Constant amounts of 1 μg of total RNA were reverse transcribed with 200 units of M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA) and Random Primers (Promega Corp.) according to the manufacturer's instructions. All investigated samples were transcribed in the same reverse transcription reaction.

The specific primers for quantitative real-time PCR were designed using publicly available sequences from the Nucleotide Sequence Database, NCBI (beta-actin: accession no. NM_001101; 18S rRNA: accession no. X03205; GAPDH: accession no. NM_002046; PBGD: accession no. X04808), or used according to literature (Neuvians et al., 2003; Pfaffl et al., 2002a): ubiquitin C forward 5′-AGATCCAGGATAAGGAAGGCAT-3′; reverse 5′-GCTCCACCTCCAGGGTGAT-3′ (198 bp); beta-actin forward 5′-AACTCCATCATGAAGTGTGACG-3′, reverse 5′-GATCCACATCTGCTGGAAGG-3′ (234 bp); 18S rRNA forward 5′-GATATGCTCATGTGGTGTTG-3′, reverse 5′-AATCTTCTTCAGTCGCTCCA-3′ (236 bp); GAPDH forward 5′-TGGTATCGTGGAAGGACTCATGAC-3′, reverse 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ (189 bp); PBGD forward 5′-AACGGCGGAAGAAAACAG-3′, reverse 5′-TCCAATCTTAGAGAGTGCA-3′ (190 bp); target gene 1 (TG 1) forward 5′-TCGCATCTCTTCTATCTGGCCCTGT-3′, reverse 5′-GCAGTACATCTCCAGCCTCCTCAGA-3′ (240 bp); target gene 2 (TG 2) forward 5′-TTAAAATGGCCAGAACCTGAG-3′, reverse 5′-ATTATAACCAAGCCTCCCAC-3′ (314 bp). A master-mix was prepared as follows: 6.4 μL water, 1.2 μL $MgCl_2$ (25 mM), 0.2 μL forward primer (20 μM), 0.2 μL reverse primer (20 μM) and 1.0 μL LightCycler® Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Nine microlitres of the master-mix was filled in glass capillaries and 1 μL PCR template containing 25 ng reverse-transcribed total RNA was added. To ensure an accurate quantification of the desired product, a high-temperature fluorescence measurement in a fourth segment of the PCR run was performed (Pfaffl, 2001). The following general real-time PCR protocol was employed: denaturation for 10 min at 95 °C, 30 cycles (beta-actin, GAPDH), 35 cycles (18S rRNA), or 40 cycles (ubiquitin C, PBGD, TG 1, TG 2) of a four-segment amplification and quantification program, a melting step by slow heating from 60 to 99 °C with a rate of 0.1 °C/s and continuous fluorescence measurement, and a final cooling down to 40 °C. The four-segment amplification and quantification program was carried out as follows: 15 s denaturation at 95 °C, 10 s annealing at 58 °C (GAPDH, 18S rRNA, PBGD), 60 °C (ubiquitin C, beta-actin), 62 °C (TG 1), 63 °C (TG 2), respectively, 15 s elongation at 72 °C and 5 s fluorescence acquisition at 83 °C (18S rRNA), 84 °C (TG 2), 85 °C (PBGD), 86 °C (beta-actin, ubiquitin C), 87 °C (GAPDH) and 88 °C (TG 1), respectively. Crossing point (CP) values were acquired by using the second derivative maximum method of the LightCycler® Software 3.5 (Roche Diagnostics). The CP is the number of PCR cycles when maximal acceleration of the fluorescence increase is reached. The earlier the fluorescence increases, the higher is the concentration of the measured mRNA. All CP of the 31 samples per investigated factor were detected in one run to eliminate interassay variance. Real-time PCR efficiencies were acquired by amplification of a standardized dilution series of the template cDNA (three replicates with readings of <0.5 CP difference) and the given slopes in the LightCycler® Software 3.5 (Roche Diagnostics). The corresponding efficiencies ($E$) were then calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2001). The specificity of the products was documented with a high-resolution gel electrophoresis and analysis of the melting temperature, which is product-specific (Pfaffl et al., 2002a). The following specific melting

temperatures were obtained: 88.35 °C (ubiquitin C), 90.14 °C (GAPDH), 89.49 °C (beta-actin), 86.15 °C (18S rRNA), 88.12 °C (PBGD), 90.37 °C (TG 1) and 87.20 °C (TG 2). PCR products were purified with the Nucleo Spin® Extraction Kit (Macherey-Nagel, Düren, Germany) and sent for commercial sequencing (GENterprise GmbH, Mainz, Germany). The results were compared to the known sequences in the NCBI database.

### 2.3. Microarray

Tissues were lyzed in TRIzol® (Invitrogen, Karlsruhe, Germany) and total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quality was controlled spectrophotometrically and by gel electrophoresis.

Microarray analyses were carried out applying 5 μg of total RNA on HG-U95Av2 microarrays (Affymetrix, Santa Clara, CA). Fragmentation of cRNA, hybridisation, washing, staining and scanning was performed according to the manufacturer's instructions. Expression values were obtained using Affymetrix™ Microarray Suite™ Version 5.0. This includes the background correction of the average of the lowest two percentiles of intensities on a four-by-four grid on the chip, the introduction of an 'ideal mismatch' forced to be lower than the corresponding perfect match, and the usage of Tukey's biweight to elicit an expression value out of single probe intensity pairs. To evaluate the quality control of starting material and efficiency of target preparation, the ratios of the-3′/5′-signal intensity of two housekeeping genes: GAPDH (probe set: AFFX-HUMGAPDH/M33197) and beta-actin (probe set: AFFX-HSAC07/X00351) were estimated. Annotation of the probe sets was taken from the annotation files provided on the Affymetrix homepage (updated version October 2003). For determination of housekeeping gene expression values the following probe sets were investigated: 35905_s_at, accession no. U34995, gene: GAPDH; 32318_s_at, accession no. X63432, gene: beta-actin; 1323_at, accession no. X04803, gene: ubiquitin B; 32153_s_at, accession no. U49869, gene: ubiquitin B; 32334_f_at, accession no. AB009010, gene: ubiquitin C; 32335_r_at, accession no. AB009010, gene: ubiquitin C; 31527_at, accession no. X17206, gene: ribosomal protein S2; 1653_at, accession no. M84711, gene: ribosomal protein S3A; 31330_at, accession no. M81757, gene: ribosomal protein S19; 2016_s_at, accession no. M64241, gene: ribosomal protein L10; 37649_at, accession no. M95623, gene: PBGD.

### 2.4. Statistical analysis

For quantitative RT-PCR investigations the statistical significance of differences in mRNA expression of the examined factors was analyzed by the Relative Expression Software Tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR (Pfaffl et al., 2002b). This software calculates an expression ratio in regard to the control group (normal testicular tissue), either normalized or not normalized by a reference gene. The expression ratio ($R$) is:

$$R = \frac{E_{\text{target}}^{\Delta\text{CP target(mean control}-\text{mean sample)}}}{E_{\text{reference}}^{\Delta\text{CP reference(mean control}-\text{mean sample)}}}$$

where $E$ is the efficiency. REST also indicates coefficients of variation (CV) in % and standard deviations based on the CPs of the target gene. The data are shown as a maximal cycle number of 40 minus the acquired CP ± S.E.M. The higher the 40 − CP values, the higher is the concentration of the target gene. As PCR amplification is an exponential process, a difference of two CP ($\Delta$CP = 2) signifies approximately a regulation by a factor of $E^2$ (with $E$ = efficiency) and is indicated in the text according to the expression ratio calculated by REST.

For microarray investigations the exploratory data analysis, Wilcoxon paired nonparametric tests for difference and the Mann–Whitney test for the nonparametric independent 2-group comparisons were performed with the program SPSS 10 for Windows (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2000 (Microsoft Corp., Redmond, WA, USA). Differences with $P \leq 0.05$ were regarded as statistically significant.

## 3. Results

### 3.1. Quantitative RT-PCR data

The investigated transcripts showed high real-time PCR efficiencies between 1.68 (GAPDH), 1.73 (TG

1), 1.75 (beta-actin), 1.81 (ubiquitin C), 1.83 (PBGD), 1.84 (18S rRNA) and 2.15 (TG 2). The mean CV% on CP base was low with values between 1.45 and 6.97% (TG 2: 1.45%, PBGD: 2.22%, 18S rRNA: 3.08%, beta-actin: 3.60%, GAPDH: 4.68%, ubiquitin C: 6.96%, TG 1: 6.97%). The real-time PCR showed a good linearity over the range of 0.2–50 ng cDNA input. The acquired CP ranged from 16.09–21.97 (GAPDH), 16.16–19.87 (beta-actin), 16.26–19.77 (ubiquitin C), 17.51–21.66 (18S rRNA), 24.36–27.46 (PBGD), 28.98–33.91 (TG 1) to 31.06–34.78 (TG 2). All investigated housekeeping genes were more strongly expressed than the selected target genes.

### 3.2. Expression of housekeeping genes

Ubiquitin C was down-regulated in seminoma by a factor of 1.9 ($P < 0.01$) (Fig. 1A) and showed no significant differences between pT1- and pT2-stages. GAPDH was up-regulated by a factor of 3.3 ($P < 0.001$) in seminoma (Fig. 1A). There were no significant differences between pT1- and pT2-stages. Beta-actin was up-regulated in seminoma by a factor of 2.4 ($P < 0.001$) (Fig. 1A). Further, beta-actin was significantly up-regulated by a factor of 1.5 ($P < 0.05$) in pT1-stages compared with pT2-stages. PBGD was up-regulated by a factor of 2.2 ($P < 0.001$) in seminoma (Fig. 1A) and showed no significant differences between pT1- and pT2-stages. There was a 3.9-fold up-regulation ($P < 0.001$) for 18S rRNA in seminoma (Fig. 2A) but no significant differences between pT1- and pT2-stages.

The geometric mean of all ($n = 5$) investigated housekeeping genes was up-regulated in seminoma by a factor of 2.4 ($P < 0.001$) (Fig. 3).

### 3.3. Normalized data

Without normalization TG 1 showed a 1.4-fold up-regulation in seminoma (expression ratio = 1.44), which was not significant ($P = 0.3$) (Fig. 3). Normalization was done by generating a ratio between target gene and reference gene (Pfaffl et al., 2002b). Normalized by the geometric mean of all housekeeping genes, TG 1 is down-regulated ($P = 0.09$) by a factor of 1.6 (expression ratio = 0.61) (Fig. 4A). Normalized by PBGD, TG 1 showed a 1.5-fold down-regulation in seminoma (expression ratio = 0.65), which was not

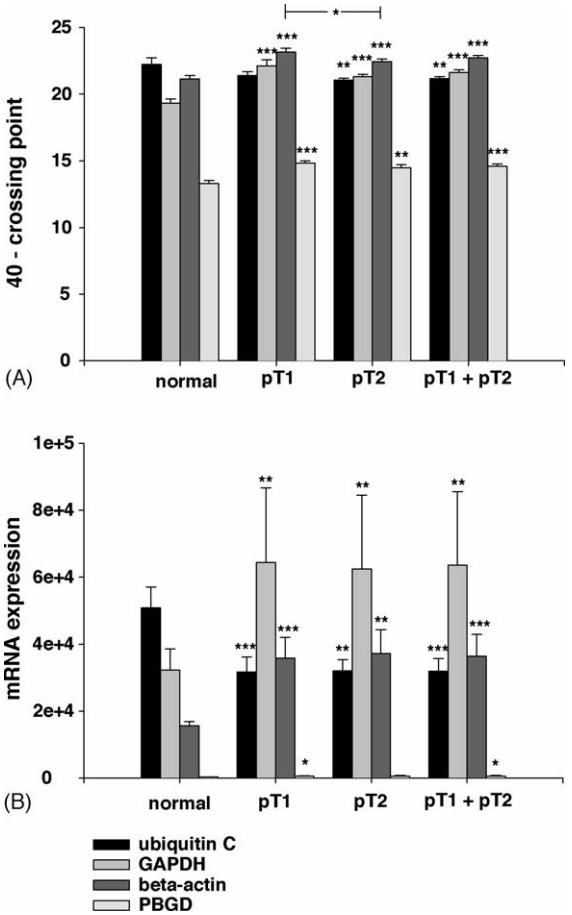


Fig. 1. Expression data (mRNA) for ubiquitin C (probe set 32334_f_at), GAPDH, beta-actin and PBGD in normal testicular tissue and pT1-, pT2- and pT1- + pT2-stages of seminoma: (A) data were acquired by real-time PCR and are shown as a maximal cycle number of 40 minus the acquired crossing point (40 − CP) ± S.E.M.; (B) data were acquired by microarray and are shown as signal intensity in counts ± standard deviation; significances are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

significant ($P = 0.2$). TG 2 was not regulated (expression ratio 1.02, $P = 0.9$) and thus constantly expressed in both groups (Fig. 3). A normalization of TG 2 by the geometric mean of all housekeeping genes results in a 2.3-fold significant down-regulation (expression ratio = 0.43, $P < 0.001$) of TG 2 in seminoma (Fig. 4B). Normalized by TG 2 the expression data of TG 1 was slightly up-regulated in seminoma by a factor of 1.4 ($P = 0.3$, expression ratio = 1.40), which is the same as in the raw expression data (Fig. 4A).

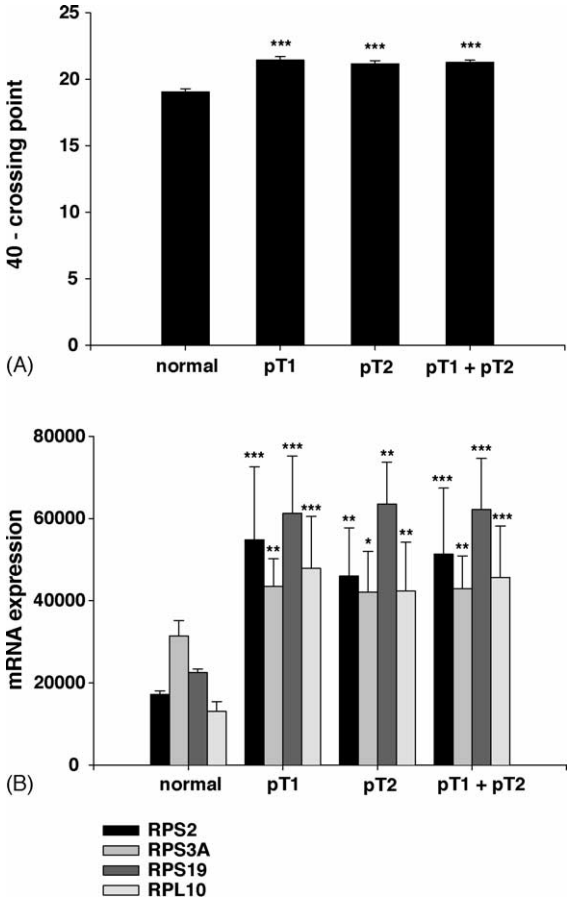


Fig. 2. Expression data for 18S rRNA (A) and the mRNA of the ribosomal proteins RPS2, RPS3A, RPS19 and RPL10 (B) in normal testicular tissue and pT1-, pT2- and pT1- + pT2-stages of seminoma: (A) data were acquired by real-time PCR and are shown as a maximal cycle number of 40 minus the acquired crossing point ($40 - CP$) ± S.E.M.; (B) data were acquired by microarray and are shown as signal intensity in counts ± standard deviation; significances are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

### 3.4. Microarray data

Seminoma showed strongly different expression patterns compared with normal testicular tissue. All investigated housekeeping genes were regulated in seminoma as presented in Figs. 1 and 2. The probe sets for GAPDH, beta-actin, PBGD (Fig. 1B) and several ribosomal proteins (RPS2, RPS3A, RPS19 and RPL10) (Fig. 2B) showed an increased mRNA expression in seminoma when compared to normal testicular tissue. Both investigated ubiquitin genes, B and C, were decreased in the testicular cancer samples (Fig. 1B). There were no significant differences between pT1- and pT2-stages of seminoma.

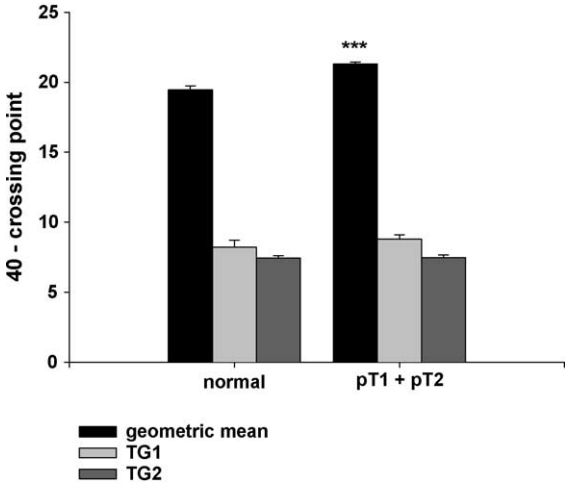


Fig. 3. Expression data (mRNA) for the geometric mean of all housekeeping genes, target gene 1 (TG 1) and target gene 2 (TG 2) in normal testicular tissue and pT1- + pT2-stages of seminoma; data were acquired by real-time PCR and are shown as a maximal cycle number of 40 minus the acquired crossing point ($40 - CP$) ± S.E.M.; significances are indicated as follows: $^{***}P < 0.001$.

## 4. Discussion

The presumed constant expression of housekeeping genes is used to normalize PCR expression data of target genes. In our study, however, normalization of the expression data with one of the commonly used housekeeping genes failed, because all of them were regulated in our sample collective. Considering the low variation of the acquired expression data (CV%: 1.45–6.97) it seems to be rather unlikely that expression differences are caused by pipetting errors or different sample input. Low expression data variation is also a prerequisite for statistically significant results, when expression differences between groups are rather low. The RNA quality of all samples was acceptable with a 28S/18S rRNA ratio of 1.7–2.0. When eliminating the samples with RNA of poorer quality (<1.8), the expression differences between seminoma and normal testicular tissue were even more concise (data not shown).

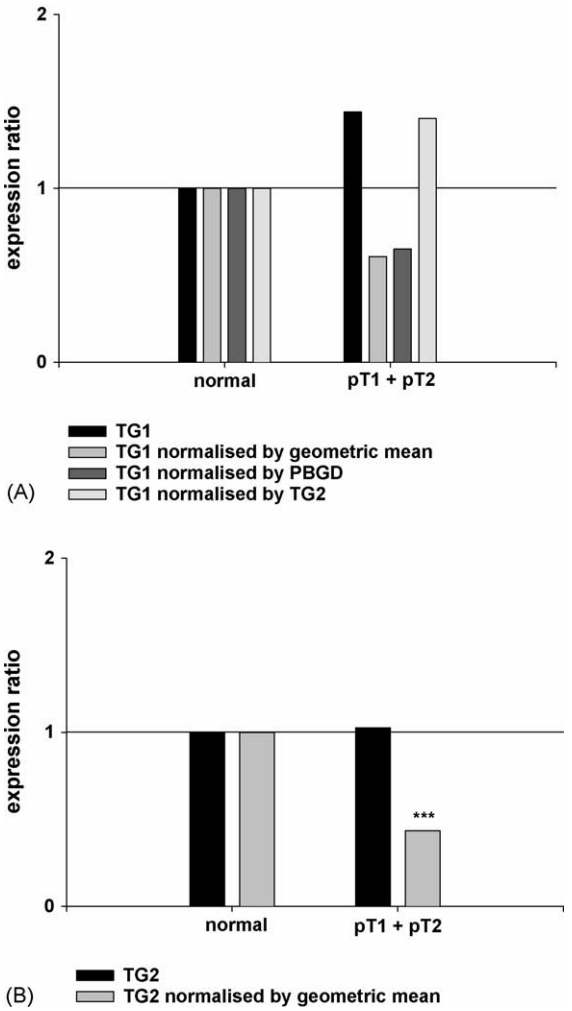


Fig. 4. Raw and normalized mRNA expression data for target gene 1 (TG 1) (A) and target gene 2 (TG 2) (B); data were acquired by real-time PCR and are shown as expression ratio in relation to normal testicular tissue (calculated according to the REST-software); an expression ratio of 1 means no regulation, >1 means up-regulation and <1 down-regulation; different reference genes were used for normalization as indicated in the diagram; significances are indicated as follows: $^{***}P < 0.001$.

A larger series of tumors, which partly included the samples investigated by quantitative PCR, could be analyzed by microarray technique. The mRNA expression of the housekeeping genes in the microarray was similar to the expression pattern in real-time PCR and thus confirmed the data. We could not compare the 18S rRNA data, since the human genome U95Av microarray chip included no probe set for ribosomal RNA. But we could verify an up-regulated mRNA expression for several ribosomal proteins, which are similar to rRNA a component of the ribosome. Looking for other unregulated housekeeping genes we found clathrin, beclin, myosin and actin gamma as possible candidate genes. On one hand, microarray analyses could be a good tool for identifying feasible reference genes. On the other hand, constantly expressed genes in the microarray are not inevitably unregulated in real-time PCR, and performing a microarray analysis before starting quantitative real-time PCR, just to find a suitable reference gene, would be very costly and spend a lot of RNA.

The normalized expression data of this study demonstrates that normalization with differentially expressed reference genes may lead to misinterpretation. The raw expression data of TG 1 shows an up-regulation in seminoma, but a down-regulation in the data, which is normalized by the geometric mean. Thus, the information of the data is inversed, even though the differences are not significant. If TG 2 is normalized by the geometric mean, the afore constantly expressed gene will be significantly ($P < 0.001$) down-regulated. This result would definitely influence the interpretation of the data. It would even make the interpretation impossible, because the investigator would have to decide subjectively, if one believes in the normalized or in the raw data. In strongly regulated genes the effect of normalization is not as important: a 16-fold down-regulation of one of our target genes (data not shown), would correspond to a 39-fold down-regulation, if normalized with the geometric mean. The sense of the interpretation of these results would be the same, only the regulation factor would be overestimated. A normalization of the target gene data with up-regulated housekeeping genes would equalize or underestimate up-regulated data and overestimate down-regulated data. TG 2 fulfils all criteria of a good reference gene: constant expression (regulation by a factor of one), unaffected by experimental conditions and similar expression level as the target genes. Normalized by TG 2, the meaning of the raw and the normalized expression data of TG 1 remains absolutely the same. The normalization does not “falsify” the main information of the original data. But if normalization does not influence the interpretation of the raw data, it is the question whether this kind of normalization is necessary at all. The commonly practiced method of searching for a

constantly expressed housekeeping gene, until luckily one was found, should be reconsidered.

A housekeeping gene should serve as an endogenous control to minimise the variation between samples. But if variation is already low in the raw data, as it can be seen in the CP-data, a normalization may no longer be needed. Soong and Ladanyi (2003) describe models for use of CP values (considering detection consistency, CP reproducibility and linear correlation to input amount) as indicators for assessing the reliability of analysis. They take CP values for more accurate indicators than conventional indicators because of their direct relationship with gene concentration.

The main standardization work should therefore be done prior to real-time PCR, which includes sample collection and transport, sample storage, sample preparation, RNA extraction, RNA quality assessment and reverse transcription. A real-time PCR performed with the same amount of good quality RNA and reverse transcribed in the same RT-step, should be accurate and reliable without any normalization. We propose the following, for comparative expression studies generally applicable standardization strategy: (a) a suitable experimental design that ensures as comparable as possible procedures of sample collection, preparation and storage; (b) strict quality control by evaluating the 28S/18S-ratio (taking into account the RNA integrity number, RIN, newly provided by Agilent Technologies) and the 260 nm/280 nm-ratio; (c) exact measurement in triplicates of total RNA; (d) the use of exactly the same amount of total RNA in the reverse transcription, performed in the same run for all investigated samples.

The investigated housekeeping genes were mainly up-regulated only ubiquitin C was down-regulated in seminoma. Considering that tumor metabolism is generally elevated because of permanent proliferation and expansion, this regulation makes sense: protein degradation is down-regulated (ubiquitin C), protein translation (18S rRNA and ribosomal proteins), energy releasing pathways (GAPDH) and cell structure elements are up-regulated (beta-actin). All housekeeping genes are more strongly expressed in pT1-seminoma than in pT2-stages, but this difference is only significant for beta-actin. pT2-stages seem to have a less active cell metabolism. But a correlation of the solely by expansion classified tumour stages and the expression data should be interpreted very carefully. A 3.9-fold regulation, as it is seen for 18S rRNA, would be preferred in target genes and not in housekeeping genes. But also housekeeping genes are genes with a specific function, and if this function is more or less demanded, their transcription may be up- or down-regulated.

In conclusion, all investigated housekeeping genes were differentially expressed in normal compared to tumor tissue and may be essential for tumor metabolism. None of the investigated housekeeping genes was suitable as a reference gene for quantitative real-time PCR in seminoma. Normalization of the expression data with one of these housekeeping genes or even with their geometric mean may lead to misinterpretation, especially in cases with low expression differences. The main standardization work should be done on study design and quantitative PCR preceding steps.

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## References

Bustin, S.A., 2000. Review: absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Fink, L., Stahl, U., Ermert, L., Kummer, W., Seeger, W., Bohle, R.M., 1990. Rat porphobilinogen deaminase gene: a pseudogene-free internal standard for laser-assisted cell picking. BioTechniques 26, 510–516.

Lupberger, J., Kreuzer, K.A., Baskaynak, G., Peters, U.R., le Coutre, P., Schmidt, C.A., 2002. Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. Mol. Cell. Probes 16, 25–30.

Neuvians, T.P., Pfaffl, M.W., Berisha, B., Schams, D., 2003. The mRNA expression of the members of the IGF-system in bovine corpus luteum during induced luteolysis. Domest. Anim. Endocrinol. 25, 359–372.

Pfaffl, M., 2001. Development and validation of an externally standardised quantitative insulin-like growth factor-1 RT-PCR using LightCycler SYBR Green I technology. In: Meuer, S., Wittwer, C., Nakagawara, K. (Eds.), Rapid Cycle Real-Time PCR, Methods and Applications. Springer Verlag, Berlin, Germany, pp. 281–292.

Pfaffl, M., Mircheva Georgieva, T., Penchev Georgiev, I., Ontsouka, E., Hageleit, M., Blum, J.W., 2002a. Real-time RT-PCR quantification of insulin-like growth factor IGF-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. Domest. Anim. Endocrinol. 22, 91–102.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002b. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Qi, L., Sit, K.H., 2000. Suicidal differential housekeeping gene activity in apoptosis induced by DCNP. Apoptosis 5, 379–388.

Rasmussen, R., 2001. Quantification on the LightCycler. In: Meuer, S., Wittwer, C., Nakagawara, K. (Eds.), Rapid Cycle Real-Time PCR, Methods and Applications. Springer Verlag, Berlin, Germany, pp. 21–34.

Soong, R., Ladanyi, A., 2003. Improved indicators for assessing the reliability of detection and quantification by kinetic PCR. Clin. Chem. 49, 973–976.

Suzuki, T., Higgins, P.J., Crawford, D.R., 2000. Control Selection for RNA Quantitation. BioTechniques 29, 332–337.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1–12.

Warrington, J.A., Nair, A., Mahadevappa, M., Tsyganskaya, M., 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiol. Genomics 2, 143–147.

Young, F.M., Illingworth, P.J., Fraser, H.M., 1998. Ubiquitin and apoptosis in the corpus luteum of the marmoset monkey (*Callithrix jacchus*). J. Reprod. Fertil. 114, 163–168.