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**Original Article**

# Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization?

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**Abstract** Using quantitative reverse transcription–polymerase chain reaction (RT-PCR), reference genes are utilized as endogenous controls for relative quantification of target genes in gene profiling studies. The suitability of housekeeping genes for that purpose in prostate cancer tissue has not been sufficiently investigated so far. The objective of this study was to select from a panel of 16 potential candidate reference genes the most stable genes for gene normalization. Expression of mRNA encoding *ACTB*, *ALAS1*, *ALB*, *B2M*, *G6PD*, *GAPD*, *HMBS*, *HPRT1*, *K-ALPHA-1*, *POLR2A*, *PPIA*, *RPL13A*, *SDHA*, *TBP*, *UBC*, and *YWHAZ* was examined in matched, microdissected malignant and nonmalignant tissue specimens obtained from 17 nontreated prostate carcinomas after radical prostatectomy by real-time RT-PCR. The genes studied displayed a wide expression range with cycle threshold values between 16 and 37. The expression was not different between samples from pT2 and pT3 tumors or between samples with Gleason scores <7 and ≥7 ($P$>0.05). *ACTB*, *RPL13A*, and *HMBS* showed significant differences ($P$<0.02 at least) in expressions between malignant and nonmalignant pairs. All other genes did not differ between the matched pairs, and the software programs geNorm and NormFinder were used to ascertain the most suitable reference genes from these candidates. *HPRT1*, *ALAS1*, and *K-ALPHA-1* were calculated by both programs to be the most stable genes covering a broad range of expression. The expression of the target gene *RECK* normalized with *HRPT1* alone and with the normalization factors generated by the combination of these three reference genes as well as with the unstable genes *ACTB* or *RPL13A* is given. That example shows the significance of using suitable reference genes to avoid erroneous normalizations in gene profiling studies for prostate cancer. The use of *HPRT1* alone as a reference

gene shown in our study was sufficient, but the normalization factors generated from two (*HRPT1*, *ALAS1*) or all three genes (*HRPT1*, *ALAS1*, *K-ALPHA-1*) should be considered for an improved reliability of normalization in gene profiling studies of prostate cancer.

**Keywords** Prostate carcinoma · Reference gene · Gene expression profiling · Relative quantification of genes · RT-PCR

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# Introduction

In gene expression studies, relative quantification is a frequently used strategy to evaluate quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) data. Instead of working with absolute concentrations based on a standard curve, target gene expression is related to a stably expressed housekeeping gene simultaneously determined in the same sample [1–3]. To be suitable as a reference gene in this commonly used approach of relative quantification, a housekeeping gene should generally fulfill several criteria [2, 4–6]. It should exhibit constitutive, nonregulated, stable expression in the samples investigated. The range of the expression should be similar to the target gene in the samples to be analyzed. The detection of the reference gene should be RNA specific; a pseudogene- and DNA-free amplification should be realized by a stringent primer design. Therefore, the selection of suitable reference genes is an important prerequisite to control the variability between clinical samples for quantitative gene expression studies with sensitive real-time PCR techniques [7, 8].

Several studies proved that the expression levels of the housekeeping genes did not remain constant under different metabolic conditions or between treatments in the same tissue, or that they showed a different behavior in various tissues [9, 10]. Nevertheless, housekeeping genes have often been adopted from the literature as reference genes without taking into account their specific tissue-dependent behavior or the special design of the respective study. Commonly known housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), β-actin (*ACTB*), and 18S ribosomal RNA (*18S-rRNA*) were used in many expression studies for different tissues [2, 4].

To find suitable reference genes for gene expression studies in prostate cancer samples, we performed a Medline search using the MeSH terms prostatic neoplasms, gene expression, and RT-PCR. We evaluated 120 articles published from August 2003 to March 2005 and found 56 articles based on the use of different reference genes: *ACTB* (20 times; 36%), *GAPD* (15 times;

27%), *18S-rRNA* (ten times; 17%), TATA box binding protein (*TBP*) (four times; 7%), β-tubulin and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) (each twice; 3.5%); ribosomal protein L13a (*RPL13A*), hydroxymethylbilane synthase (*HMBS*), and peptidylprolyl isomerase A (*PPIA*) (each once; 1.7%). This search clearly shows that (a) an unequivocal view of a reference gene for prostate cancer does not currently exist; (b) *ACTB* and *GAPD* have been very frequently used for normalization in prostate cancer research [11–14], although the issue of these genes for normalization has been raised [15]; and (c) meaningful studies on the application of reference genes for prostate cancer research have not been performed until now. Therefore, the aims of our study were (a) to investigate a panel of 16 frequently used housekeeping genes with regard to their suitability as reference genes for gene expression studies in paired malignant vs nonmalignant prostate tissue samples and (b) to select from this panel appropriate genes for the normalization procedure at different expression levels. For that purpose, only RNA samples with high quality and integrity characterized by Agilent technology were examined [16], and the selection of suitable reference genes was objectified by the use of two free software available on the Internet [5, 17, 18]. The significance of suitable reference genes for normalization was demonstrated using the target gene reversion-inducing, cysteine-rich protein with Kazal motifs (*RECK*) as an example.

# Materials and methods

## Patients and samples

Prostate tissue samples from 17 patients (mean age 61 years, range 47–70 years; mean preoperative concentration of prostate-specific antigen 11.6 μg/l, range 5–32 μg/l) undergoing radical prostatectomy at the Department of Urology of the University Hospital Charité were used. Tumor stage was determined according to the International Union Against Cancer [19], while tumor grading was done according to Gleason [20]. Eleven of the 17 tumors were classified as stage pT2, and the other six as stage pT3. The Gleason score was as follows: two times Gleason sum 5, four times Gleason sum 6, eight times Gleason sum 7, and three times Gleason sum 8. Five patients showed positive surgical margins (R1) but no one had metastases (M0 and pN0). The use of the tissue material for research was approved by the ethics commission of the Charité Hospital.

The prostatectomy specimens were sectioned immediately after surgical removal. Suitable tissue pieces were snap-frozen in liquid nitrogen and stored at −80°C until further processing. To obtain homogeneous and histologically well-characterized samples for mRNA analyses, we performed

microdissection as described previously [21]. Briefly, frozen tissue samples were cut into 8-µm-thick slices and were mounted on glass slides (Menzel Glaeser, Braunschweig, Germany). The slides were shortly stained with hematoxylin and aqueously mounted. To obtain matched pairs of samples for microdissection, histopathological evaluation and selection of malignant and nonmalignant areas of the tissue sections from the same prostate were performed by an experienced pathologist (G.K.). The tissue areas, manually scraped (dissected) with hypodermic needles from 20 consecutive slides under a microscope, were collected into a tube with 350 µl RNA lysis/binding buffer of the RNeasy Mini Kit (Qiagen, Hilden, Germany) including 1% beta-mercaptoethanol. With this technique, 17 cancer lesions were isolated: three Gleason grade 2, eight Gleason grade 3, five Gleason grade 4, and one Gleason grade 5.

## RNA isolation and characterization

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) based on a silica gel membrane technology by selective binding, stepwise washing, and elution of RNA following manufacturer's instructions. A digestion step on the spin column with DNase I was performed. The concentration of the isolated RNA and the ratio of absorbance at 260 nm to 280 nm ($A_{260}/A_{280}$ ratio) were measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). The accuracy of the measurements on the NanoDrop spectrophotometer was previously controlled by comparative measurements using the conventional UV spectrophotometer Ultrospec 3000 (Pharmacia). The slope of the regression line of the concentrations measured with both methods did not differ ($Y_{\text{conc.Ultrospec}}=1.021\times X_{\text{conc.NanoDrop}}+1.99$; $n=19$; $r=0.998$). These results showed the equality of RNA measurements with both methods and confirmed the accuracy of RNA measurements on the NanoDrop spectrophotometer. The precision of the RNA measurements was controlled by 12 repeated measurements of two self-prepared RNA control materials with lower and higher concentrations. The intraserial precision given as coefficient of variation resulted in 0.60% for the lower concentration (92.9±0.56 ng/µl) and 0.34% for the higher concentration (841.6±2.84 ng/µl).

The integrity of RNA was assessed with the RNA 6000 Nano LabChip kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The electropherograms and gel-like images were evaluated with the Agilent 2100 Expert software that generates the RNA integrity number (RIN) to characterize RNA integrity. This number describes a gradual scale of RNA integrity from 1 (RNA completely degraded) to 10 (RNA without degradation), taking into account

not only the conventional ratio of 28S to 18S ribosomal RNA (rRNA) but also other critical regions of the entire RNA electropherogram [22, 23].

The threshold inclusion values for the RNA samples were >1.90 for the $A_{260}/A_{280}$ ratio and >7 for the RIN value.

## First-strand cDNA synthesis

One microgram RNA was reverse transcribed using the Omniscript Reverse Transcriptase Kit (Qiagen) for first-strand cDNA synthesis for two-tube RT-PCR with 10 μM $p(dN)_8$ random primers (TIB MOLBIOL, Berlin, Germany). Before transcription, RNA was denatured for 5 min at 65°C followed by cooling on ice. Finally, the Omniscript Reverse Transcriptase was inactivated by heating the reaction mixture for 5 min at 93°C. cDNA was stored at −20°C until RT-PCR analysis. Each RNA sample was controlled for genomic DNA contamination by a reaction mix without reverse transcriptase addition. All cDNAs were diluted 1:5 before being used as PCR template.

## Real-time RT-PCR

Two real-time PCR instruments were used for measurements. The ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for the genes *ACTB*, albumin (*ALB*), beta-2-microglobulin (*B2M*), *GAPD*, glucose-6-phosphate dehydrogenase (*G6PD*), K-alpha-1 tubulin (*K-ALPHA-1*), *PPIA*, polymerase (RNA) II (DNA-directed) polypeptide A 220 kDa (*POLR2A*), *RPL13A*, succinate dehydrogenase A complex (*SDHA*), *TBP*, ubiquitin C (*UBC*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (*YWHAZ*), whereas the LightCycler Instrument (Roche Applied Science, Mannheim, Germany) was applied for *ALAS1*, *HPRT1*, and *HMBS*. Essential gene-specific data, including measurement details like primers, probes, amplicon sizes, and PCR efficiencies, are given in Table 1.

**[Table 1 will appear here. See end of document.]**

The measurements on the ABI Prism System were performed with primers and TaqMan probes as previously described [6] except for *SDHA* and *UBC* with newly designed primers and probes (Table 1). The TaqMan probes were 5′-labeled with the reporter fluorescent dye 6-carboxy-fluorescein (FAM) and carried the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) labeled on a thymidine base near the 3′ terminus. The amplification procedures were performed under the same reaction conditions as previously described in detail [6]. Briefly, the reaction mixture consisted of 10× PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], 4.5 mM $MgCl_2$, 1 mM dNTP, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany),

0.2 μM each primer, 120 nM specific TaqMan probe, and 1 μM 6-carboxy-X-rhodamine (Molecular Probes, Leiden, Netherlands). Two microliters of prediluted cDNA was used as template for the PCR in a final volume of 25 μl. The cycle conditions were set as follows: start with 3 min template denaturation at 94°C, 45 cycles of denaturation at 94°C for 20 s, and combined primer annealing/elongation at the gene-specific primer temperature for 30 s as described [6]. The annealing/elongation temperature for both *SDHA* and *UBC* was 65°C for 30 s.

The mRNAs of *ALAS1*, *HPRT1*, and *HMBS* were quantified by ready-to-use Housekeeping Gene Sets (Roche) on the LightCycler Instrument. These kits include gene-specific primers and hybridization probes as well as RNA standards. The amplifications were performed with the LightCycler-FastStart DNA Master$^{PLUS}$ Hybridization Probes according to the manufacturer's instructions (Roche). The final reaction volume of 20 μl included 1 μl prediluted cDNA as template. The cycle conditions were set as follows: a preincubation step for 10 min at 95°C for *Taq* DNA polymerase activation, followed by 45 cycles each for 15 s at 95°C for template denaturation, at 55°C for primer annealing, and at 72°C for primer extension. The temperature transition rate was 20°C/s and the PCR runs were finished after 45 cycles.

For the quantitative comparison of amplification rates of the investigated candidate reference genes, the "threshold cycles" ($C_T$ values; ABI Prism 7700) or "crossing points" ($C_P$ values; LightCycler) were used. Both values define the fluorescence signal point where a background fluorescence is exceeded. The $C_T$ value (this is synonymously used with $C_P$ value in succeeding paragraphs) correlates inversely to the cDNA concentration and depends on PCR efficiency. The PCR efficiency was determined for each gene by dilutions of plasmids ($10^6$–$10^1$ copies) for methods running on the ABI Prism 7700 or of RNA standards included in the assay kit (Roche) for LightCycler methods. The PCR efficiencies calculated according to Rasmussen [24] are given in Table 1.

*RECK* (accession number NM_021111) was measured as target gene on the LightCycler instrument using the QuantiTect SYBR Green PCR Kit (Qiagen) with the following primer pair: forward, 5′-CCCAGATTATTGCCCAGAGA; reverse, 5′-GCAATAGCCAGTTCACAGCA. The PCR efficiency was 1.98; the amplicon size was 120 bp.

Controlled and permanently maintained pipetting devices (Eppendorf AG, Hamburg, Germany) were used for all measurements. Duplicate measurements were performed and mean values were calculated. In addition, to minimize variability of measurements significant for the comparisons between tumor and nontumor samples, the cancerous and noncancerous samples were always analyzed as paired samples in one analytical run to exclude between-run variations. The analytical

performance of the measurements was characterized by within-run controls with high and low concentrations of cDNA for *GAPD* (*n*=10) and *HMBS* (*n*=11) amplifications corresponding to mean $C_T$ values of 19.79 and 31.42, and 21.89 and 31.59, respectively. The coefficients of variation were 0.41 and 0.65% for *GAPD* measurements and 0.15 and 0.59% for *HMBS* measurements.

## Data analysis

Statistical analyses were performed with GraphPad Prism for Windows, version 4.03 (GraphPad Software, San Diego, CA, USA). The distribution fitting procedure according to the D'Agostino–Pearson omnibus normality test and both paired and unpaired Student's *t* tests were applied. P values <0.05 were considered statistically significant.

For stability comparison of candidate reference genes, we applied the software geNorm, version 3.4 [5], and NormFinder [18]. The program geNorm is a Visual Basic application tool for Microsoft Excel and is available on the Internet upon request by the programmers. $C_T$ values were converted into relative quantities for analysis with geNorm, considering the PCR efficiencies of the genes as shown in Table 1 [5]. The program selects from a panel of candidate reference genes the two most stable genes or a combination of multiple stable genes for normalization. The NormFinder is also freely available on the Internet ( http://www.mdl.dk). It is a Microsoft Excel add-in and calculates the stability values of the individual candidate reference genes for normalization [18]. The stability value is based on the combined estimate of intra- and intergroup expression variations of the genes studied. A low stability value indicating a low combined intra- and intergroup variation proves high expression stability. Using this approach, the most stable single gene is calculated and an additional combination of two genes is recommended because the stability value of that combination is generally lower than that of the single gene.

# Results

## RNA quality

All RNA samples were examined as to their concentration, purity, and integrity. Based on the absorbance ratio at 260 nm to 280 nm (mean±SD, 2.00±0.05), all RNA samples were pure and protein free. The RNA integrity was assessed by the calculation of RIN values using the Agilent 2100 Bioanalyzer. The matched malignant and nonmalignant tissue samples revealed RIN values

(mean±SD) of 7.9±0.23 and 7.7±0.14, respectively, which were not significantly different (paired *t* test; *P*=0.47).

## Expression levels of candidate reference genes

The 16 housekeeping genes studied displayed a wide expression range, with $C_T$ values between 16 and 37. The expression was not different between samples from pT2 and pT3 tumors, between samples obtained from patients with Gleason scores <7 and ≥7, or between samples from Gleason grade ≤3 and ≥4 lesions (*t* test; *P*>0.05). All genes except *POLR2A* showed a normal distribution pattern proved by the D'Agostino–Pearson fitting procedure both in the malignant and nonmalignant tissue samples. We grouped the expression levels in three arbitrary ranges as marked in Fig. 1. The nonmalignant and the malignant samples were separately shown as box plots with ranges as whiskers to demonstrate the total expression ranges. Highly expressed genes with $C_T$ values below 20 cycles were *ACTB*, *GAPD*, *RPL13A*, and *UBC*. Genes with low expression and $C_T$ values above 28 cycles were *ALAS1*, *ALB*, *HMBS*, and *HPRT1*. Intermediately expressed genes with $C_T$ values between 20 and 28 cycles were *B2M*, *G6PD*, *K-ALPHA-1*, *POLR2A*, *PPIA*, *SDHA*, *TBP*, and *YWHAZ*.

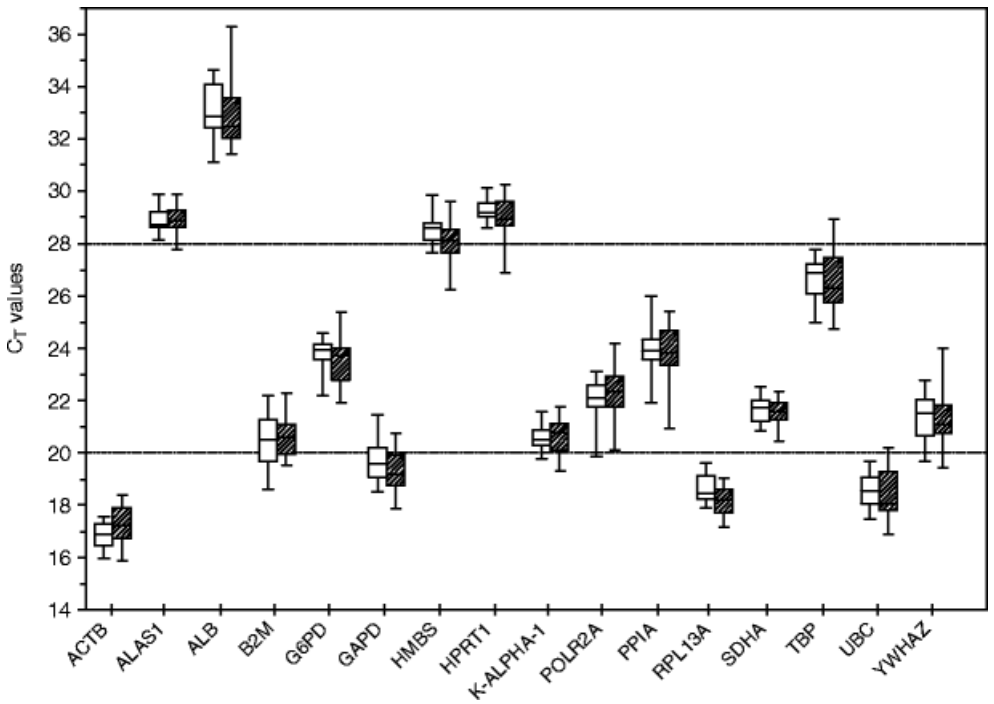


**Fig. 1** Expression levels of candidate reference genes in malignant and nonmalignant prostate samples. Values are given as real-time PCR cycle threshold numbers ($C_T$ values). *Boxes* (*blank*, nonmalignant; *cross-striated*, malignant) represent the lower and upper quartiles with medians; *whiskers* represent the

ranges for the data of 17 matched samples. The *arbitrary lines* at $C_T$ 20 and 28 distinguish three groups of differently expressed housekeeping genes

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The individual housekeeping genes have different expression ranges over all studied samples shown in Fig. 1. Genes spanning a maximum expression range of more than about four to five cycles were *ALB* (range 5.16), *PPIA* (range 5.07), *YWHAZ* (range 4.59), and *TBP* (range 4.18). Genes with smallest expression ranges of about two cycles were *SDHA* (range 2.12), *ALAS1* (range 2.15), and *K-ALPHA-1* (range 2.46).

Using the paired Student's *t* test, significant differences in gene expression between malignant and nonmalignant sample pairs were observed only for *ACTB* (paired *t* test; $P$=0.010), *RPL13A* ($P$=0.013), and *HMBS* ($P$=0.009). The differences prove that these genes are unsuitable reference genes to be used for normalization purposes in gene profiling studies between normal and malignant prostatic tissue. These genes were consequently excluded from subsequent calculations in geNorm and NormFinder.

## Expression stability of candidate reference genes and normalization approach

From the theoretical point of view, the remaining 13 housekeeping genes without expression differences in malignant and nonmalignant tissue samples are all suitable reference genes for normalization between nonmalignant and malignant prostate tissue. However, in search of the most stable reference genes and in order to restrict the number of genes, the gene expression stability was validated with the two software programs, geNorm and NormFinder [5, 17, 18].

The program geNorm calculates the gene expression stability measure *M* of one gene based on the average pairwise variation between all studied genes. The lowest *M* values characterize genes with the most stable expression. Successive elimination of the least stable gene generates a ranking of genes according to their *M* values and results in the identification of the two most stable genes. The average expression *M* values of the 13 candidate reference genes are plotted in Fig. 2a. All genes studied achieved high expression stability with low *M* values less than 0.9 that were below the default limit of 1.5 in the geNorm program. The curve represents the stepwise exclusion of the least stable housekeeping gene. *ALB* and *UBC* were at first excluded as the least stable genes. *ALAS1* and *HPRT1* (both $M$=0.30) were identified as the two most stable genes.

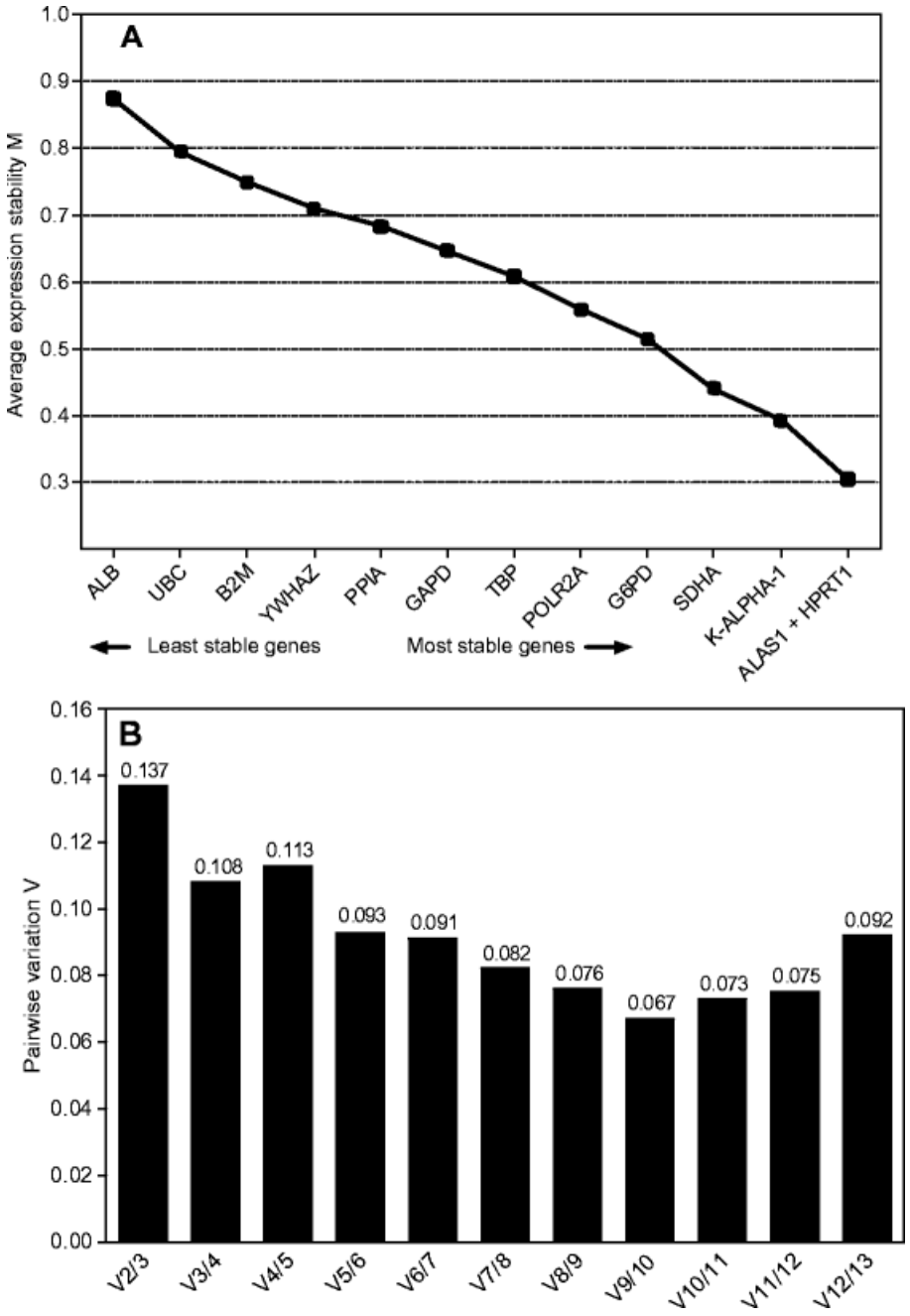


**Fig. 2** Selection of the most suitable reference genes for normalization in prostate cancer samples using geNorm analysis. To identify the best-performing reference genes, we performed geNorm analysis of the remaining 13 genes after the exclusion of the unstable genes *ACTB*, *RPL13A*, and *HMBS* (for further details, see text), and the results are presented according to the output file of the program [5, 17]. **a** Stepwise exclusion of the least stable genes by calculating the average expression stability measure *M*. The value of *M* was calculated for each gene, and the least stable gene with the highest *M* value was automatically excluded for the next calculation round. The *x-axis* from left to right indicates the ranking of the genes according to their expression stability. **b** Determination of the optimal number of reference genes for normalization. The software calculates the normalization factor from at least two genes at which the variable *V* defines the

pairwise variation between two sequential normalization factors. For example, *V*3/4 shows the variation of the normalization factor of three genes in relation to four genes. For further details, see text

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The geNorm program calculates, in addition to the gene-stability measure *M*, a normalization factor assessing the optimal number of reference genes for generating that factor (Fig. 2b). The normalization factor is calculated from at least two genes taking into account the variable *V* as the pairwise variation between two sequential normalization factors [5, 17]. It is remarkable that, being the two most stable housekeeping genes, *ALAS1* and *HPRT1* yielded a *V* value of 0.137, which is less than 0.15. That cutoff was suggested as the limit beneath which it would not be necessary to include additional reference genes for normalization. Since the *V* value did not essentially decrease when more than four reference genes were included, it can be concluded that normalization using either two (*ALAS1*, *HPRT1*) or three (*ALAS1*, *HPRT1*, *K-ALPHA-1*) reference genes is an adequate normalization approach for gene profiling studies in prostate cancer.

Table 2 shows the ranking order of the 13 candidate reference genes mentioned above using the other program, the NormFinder, to calculate their expression stability. Again, the genes *HPRT1*, *ALAS1*, and *K-ALPHA-1* were found to be the most stable genes. The NormFinder selected *HPRT1* with a stability value of 0.031 as the most stable single gene. The best combination of two genes was that of *HRPT1* and *ALAS* (Table 2), which improved the stability value to 0.027, indicating a more reliable normalization [18]. As shown above, that combination corresponded to the final combination of two genes calculated by the geNorm program.

**[Table 2 will appear here. See end of document.]**

## Significance of suitable reference genes for normalization

To demonstrate the significance of suitable reference genes for normalization in order to get correct profiling data, we measured *RECK* mRNA in 14 paired malignant and nonmalignant prostate cancer tissue samples. The normalized *RECK* expression was performed using five different strategies: approaches with two (*HPRT1*, *ALAS1*) or three (*ALAS1*, *HPRT1*, *K-ALPHA-1*) reference genes calculated by NormFinder and geNorm, respectively, with the top-ranked reference gene *HPRT1* recommended by the NormFinder and with the two unstable genes *ACTB* and *RPL13A* eliminated in the first step of the described procedure to identify stable reference genes. We used *ACTB* and *RPL13A* as examples of unstable genes because *ACTB* was decreased and *RPL13A* was increased in malignant compared with the nonmalignant samples (Fig. 1). Normalization with the approaches recommended by geNorm and NormFinder similarly showed significantly reduced

*RECK* expression by about 25% in tumor samples (Fig. 3). In contrast, normalization using *ACTB* resulted in *RECK* expression in tumor samples with a tendency to be higher by about 10% compared with noncancerous samples, while the use of *RPL13A* showed a significantly higher decrease (by about 40%) in tumor samples. These contrary results using the two unstably expressed genes for normalization in comparison with the selected stable reference genes can be explained by the inverse expression of *ACTB* and *RPL13A* in cancerous and noncancerous samples. This example alone supports the particular significance to draw special attention to the selection of reference genes for normalization of RT-PCR data. (Raw data are available at http://www.charite.de/ch/uro/de/html/arzt_forschung/hkg.)

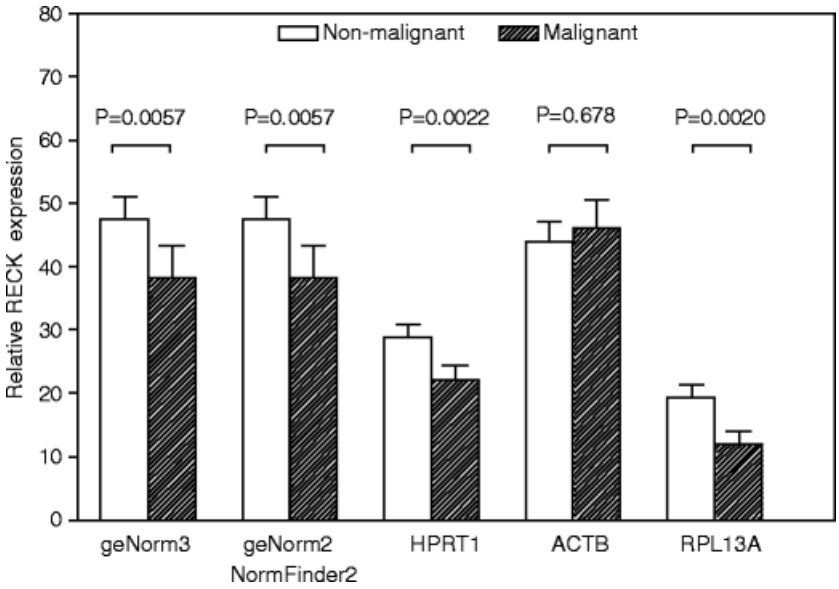


**Fig. 3** Effect of different normalization approaches on the expression levels of *RECK* in malignant and nonmalignant prostate tissue samples. RT-PCR measurements of *RECK* and the corresponding reference genes were performed in 14 pairs of samples. Normalizations were made under different conditions as indicated: geNorm3, with the normalization factor calculated from the genes *ALAS1*, *HPRT1*, and *K-ALPHA-1* using the program geNorm; geNorm2 and NormFinder2, normalization by the genes *ALAS1* and *HPRT1* as the best combination of two genes calculated by both programs; *HPRT1*, *ACTB*, and *RPL13A*, use of these genes alone. Normalized *RECK* expression values are given as mean values±SEM; differences between the normalized RECK expression in nonmalignant (*blank bars*) and malignant (*cross-striated bars*) samples were calculated by the Student's *t* test of paired data

# Discussion

To the best of our knowledge, the present study is the first systematic comparison of a large number of potential reference genes with regard to their utility as normalizers in prostate cancer samples. There are only two articles about reference gene evaluations for prostatic tissue [6, 25]. However, those studies were performed using either commercially available cDNA [6] or RNA samples obtained from a small number of unpaired bulk tissue specimens [25]. Other studies, as briefly mentioned in the "Introduction," used different reference genes for relative quantification in prostate cancer samples, but their usefulness as normalizers was not always as strictly examined as in the present study.

All our experimental data and the results arising from subsequent calculations are based on the particular design of our study, which is characterized by the following five features: (1) use of matched malignant and nonmalignant specimens from the same prostate, (2) use of microdissected samples instead of bulk tissue, (3) stringent control of the isolated RNA, (4) simultaneous investigation of a large number of 16 potential reference genes, and (5) use of a software combined with a preceding *t* test to rank the candidate genes regarding their suitability as normalizers. It is necessary to discuss some of these characteristics because they were significant for the reliability of data and the conclusions derived from them.

In addition to tissue storage and preparation conditions as crucial prerequisites for reliable expression results [16], we particularly paid attention to the first three characteristics to avoid preanalytical confounding factors. Matched pairs of malignant and nonmalignant samples instead of unpaired samples were used to minimize interindividual variations and thus to increase the power of data analysis. We showed that the expression of the examined candidate reference genes was affected neither by tumor stage nor by grade. That is an important precondition and denotes both a time- and cost-saving advantage of using the selected genes as normalizers in all malignant samples independent of tumor stage or grade. This is in contrast to the expression of target genes that often show a stage- or grade-dependent differential gene expression. Since prostate cancer lesions are often heterogeneously distributed in the prostate, the use of bulk tissue from prostatectomy specimens is scarcely appropriate for detailed analysis. To obtain homogeneous and histologically characterized samples, we used microdissected samples [21].

In addition to these two criteria of tissue selection, only RNA samples with high purity and integrity showing an $A_{260}/A_{280}$ ratio >1.90 and an RIN >7.0 were included in this study. Our special attention was directed to the control of RNA integrity by the Agilent 2100 Bioanalyzer [7, 8],

including a new expert software program for calculating the RINs [22]. That system allows for a more sensitive analysis from a low RNA yield compared to 28S to 18S rRNA band ratio by gel electrophoresis [22] and warrants a precise control of RNA quality in all samples to obtain reliable expression results. This method was recently recommended as the standard operational procedure for RNA quality assessment and was considered to be the state of the art in facilitating comparisons of downstream experiments [26]. Therefore, taking into account this important reason, we decided to use total RNA instead of mRNA in our experiments from small microdissected samples in order to have the possibility to characterize the integrity of RNA by this technique. That was the essential precondition to use samples with comparable quality criteria. These stringent quality data cannot be established with mRNA because only a visual inspection of the electropherogram of Agilent Bioanalyzer is possible but no metric data are available. Thus, the conclusions drawn from our experiments regarding the selection of suitable reference genes are, strictly speaking, only relevant if total RNA is used for gene profiling studies.

The use of the so-called housekeeping genes for normalization is a constant matter of debate [4, 15] with numerous pros and cons [2, 15, 27]. As already mentioned, conflicting results concerning the stability of the same housekeeping genes were described. Reasons for these discrepancies are not always clearly apparent even if the same tissue was investigated. One reason might be the rather equivocal assumption of the general stability of housekeeping genes. However, one should note that the expression levels of a given gene used as reference gene can differ not only between various tissues but also in the same tissue under different conditions [28]. To prove whether a candidate gene is suitable for the normalization of gene expression, it is necessary to define the problem to be investigated. Differential gene studies in treated compared with untreated patients, older vs younger patients, or male vs female patients should consider that not only target genes could vary under these conditions but housekeeping genes as well. In addition, it has to be decided whether the expression between normal and tumor samples in general or only among different stages and/or grades should be compared. The basic requirement of a candidate gene to be used for normalization purposes is its nondifferent expression in the respective study groups. Thus, a specific validation of potential reference genes for the respective condition is needed.

Our approach included two steps. In the first step, according to our study design of matched malignant and nonmalignant samples, candidates of reference genes were evaluated regarding their expression levels, and the genes with significantly different expression levels between the two groups had to be excluded. Three genes (*ACTB*, *RPL13A*, and *HMBS*) out of the 16 potential reference genes studied significantly differed in their expression, as indicated by the cycle threshold

values in nonmalignant and malignant tissue samples. It could be concluded that these genes are regulated and they are not dedicated for target gene normalization in prostate cancer samples. We consider this initial calculation as mandatory to exclude these genes from further calculations because the programs geNorm and NormFinder are unable to detect them. In the second step, the best-performing genes or combinations of genes for normalization were determined using the software programs geNorm as well as NormFinder [5, 18]. The search for suitable housekeeping genes is both time-consuming and cost-intensive, and various programs and methods have been suggested to simplify the housekeeping gene search [29–31]. The software programs geNorm and NormFinder have been used in other studies to find suitable reference genes from a set of candidates [7, 18, 32–36]. In our study, both programs equally identified *HPRT1*, *ALAS1*, *K-ALPHA-1*, and *SDHA* as the most stably expressed genes. These genes represent the three arbitrarily defined levels of low, intermediate, and high gene expression (Fig. 1). Thus, for studying target gene profiling in prostate cancer tissue with different expression levels, appropriate reference genes in a range similar to the target genes are available for normalization. Recently, *HPRT1* was recommended as a universal, single reference gene for differential expression studies in cancer research [15].

However, normalization using only one reference gene was suggested to be replaced by normalization based on the multiple, best-performing candidate reference genes [5, 17]. A normalization factor including more than one reference gene has the benefit of more accurate normalization calculation than a single gene and was also suggested by others [7, 27, 36], especially in situations when no optimal reference gene has been available [18]. In our study, using one reference gene (*HRPT1*, calculated by the software NormFinder) yielded similar normalization data demonstrated for the example of *RECK* mRNA expression, like the application of normalization factors calculated from two (*HRPT1*, *ALAS1*) or three (*HRPT1*, *ALAS1*, *K-ALPHA-1*) genes (Fig. 3). It might be that our optimal study conditions resulting in high-quality RNA samples made only one reference gene necessary. Further experiments on this problem are essential.

The example of *RECK* mRNA expression depending on the normalization approach used (Fig. 3) demonstrates the significance of reference genes to obtain reliable expression data. *RECK* is a membrane-anchored regulator of matrix metalloproteinases implicated in tumor angiogenesis [37]. Without going into more detail regarding the possible importance of *RECK* for prostate cancer, the comparison of the relative expressions shows that *ACTB* along with *GAPDH*, most frequently used in gene profiling studies of prostate carcinoma [11–13] even by us [38, 39], must be considered as unsuitable for normalization in comparison to the other genes studied. Similar erroneous

normalizations resulted in other tissues like breast cancer or cell lines when inadequate reference genes or normalizing strategies were applied [5, 7, 36].

In conclusion, the three most stable genes *HPRT1*, *ALAS1*, and *K-ALPHA-1* covering a broad expression range can be used as reference genes for relative gene quantification and normalization purposes in gene profiling studies of prostate cancer. The use of *HPRT1* alone as reference gene shown in our study was sufficient, but the normalization factors generated from two (*HRPT1*, *ALAS1*) or all three genes (*HRPT1*, *ALAS1*, *K-ALPHA-1*) should be considered for an improved reliability of normalization.

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# References

1. Karge WH, III, Schaefer EJ, Ordovas JM (1998) Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. Methods Mol Biol 110:43–61
2. Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193
3. Suzuki T, Higgins PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques 29:332–337
4. Thellin O, Zorzi W, Lakaye B, De Borman BB, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 75:291–295
5. 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:research0034.1–research0034.11
6. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313:856–862
7. Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA, Orlando C (2002) Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 309:293–300
8. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 37:112–119
9. Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69–81
10. Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 295:17–21
11. Nagler DK, Kruger S, Kellner A, Ziomek E, Menard R, Buhtz P, Krams M, Roessner A, Kellner U (2004) Up-regulation of cathepsin X in prostate cancer and prostatic intraepithelial neoplasia. Prostate 60:109–119

12. Katenkamp K, Berndt A, Hindermann W, Wunderlich H, Haas KM, Borsi L, Zardi L, Kosmehl H (2004) mRNA expression and protein distribution of the unspliced tenascin-C isoform in prostatic adenocarcinoma. J Pathol 203:771–779
13. Jung C, Kim RS, Zhang HJ, Lee SJ, Jeng MH (2004) HOXB13 induces growth suppression of prostate cancer cells as a repressor of hormone-activated androgen receptor signaling. Cancer Res 64:9185–9192
14. Tian W, Osawa M, Horiuchi H, Tomita Y (2004) Expression of the prolactin-inducible protein (PIP/GCDFP15) gene in benign epithelium and adenocarcinoma of the prostate. Cancer Sci 95:491–495
15. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39
16. Almeida A, Paul TJ, Magdelenat H, Radvanyi F (2004) Gene expression analysis by real-time reverse transcription polymerase chain reaction: influence of tissue handling. Anal Biochem 328:101–108
17. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2004) GeNorm software manual, update 6 Sep 2004. http://medgen.ugent.be/~jvdesomp/genorm
18. Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250
19. Sobin LH, Wittekind C (2002) TNM classification of malignant tumours, 6th edn. Wiley-Liss, New York, pp 184–187
20. Gleason DF (1988) Histologic grade, clinical stage, and patient age in prostate cancer. NCI Monogr 7:15–18
21. Kristiansen G, Pilarsky C, Wissmann C, Stephan C, Weissbach L, Loy V, Loening S, Dietel M, Rosenthal A (2003) ALCAM/CD166 is up-regulated in low-grade prostate cancer and progressively lost in high-grade lesions. Prostate 54:34–43
22. Mueller O, Lightfoot S, Schroeder A (2004) RNA integrity number (RIN)—standardization of RNA quality control. Agilent Technologies, Palo Alto
23. Miller CL, Diglisic S, Leister F, Webster M, Yolken RH (2004) Evaluating RNA status for RT-PCR in extracts of postmortem human brain tissue. Biotechniques 36:628–633
24. Rasmussen R (2001) Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K (eds) Rapid cycle real-time PCR, methods and applications. Springer, Berlin Heidelberg New York, pp 21–34
25. de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest 85:154–159
26. Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C (2005) Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Res 33:e56
27. Schmid H, Cohen CD, Henger A, Irrgang S, Schlondorff D, Kretzler M (2003) Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. Kidney Int 64:356–360
28. Nelson PS (2004) Predicting prostate cancer behavior using transcript profiles. J Urol 172:S28–S32
29. Haller F, Kulle B, Schwager S, Gunawan B, von Heydebreck A, Sultmann H, Fuzesi L (2004) Equivalence test in quantitative reverse transcription polymerase chain reaction: confirmation of reference genes suitable for normalization. Anal Biochem 335:1–9
30. Szabo A, Perou CM, Karaca M, Perreard L, Quackenbush JF, Bernard PS (2004) Statistical modeling for selecting housekeeper genes. Genome Biol 5:R59
31. Janssens N, Janicot M, Perera T, Bakker A (2004) Housekeeping genes as internal standards in cancer research. Mol Diagn 8:107–113
32. Kim S, Kim T (2003) Selection of optimal internal controls for gene expression profiling of liver disease. Biotechniques 35:456–458 (460)
33. Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H (2003) Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. J Med Microbiol 52:403–408

34. Biederman J, Yee J, Cortes P (2004) Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis. Kidney Int 66:2308–2314

35. Ontsouka EC, Reist M, Graber H, Blum JW, Steiner A, Hirsbrunner G (2004) Expression of messenger RNA coding for 5-HT receptor, alpha and beta adrenoreceptor (subtypes) during oestrus and dioestrus in the bovine uterus. J Vet Med A Physiol Pathol Clin Med 51:385–393

36. Hoerndli FJ, Toigo M, Schild A, Gotz J, Day PJ (2004) Reference genes identified in SH-SY5Y cells using custom-made gene arrays with validation by quantitative polymerase chain reaction. Anal Biochem 335:30–41

37. Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM, Nishimura S, Imamura Y, Kitayama H, Alexander DB, Ide C, Horan TP, Arakawa T, Yoshida H, Nishikawa S, Itoh Y, Seiki M, Itohara S, Takahashi C, Noda M (2001) The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. Cell 107:789–800

38. Jung M, Römer A, Keyszer G, Lein M, Kristiansen G, Schnorr D, Loening SA, Jung K (2003) mRNA expression of the five membrane-type matrix metalloproteinases MT1–MT5 in human prostatic cell lines and their down-regulation in human malignant prostatic tissue. Prostate 55:89–98

39. Stephan C, Yousef GM, Scorilas A, Jung K, Jung M, Kristiansen G, Hauptmann S, Bharaj BS, Nakamura T, Loening SA, Diamandis EP (2003) Quantitative analysis of kallikrein 15 gene expression in prostate tissue. J Urol 169:361–364

**Table 1** Characteristics of gene-specific real-time PCR assays

| Gene symbol | Gene name | Accession no. | Primer/Probe sequence [5′→3′][a] | Amplicon size (bp) | PCR efficiency[b] |
|---|---|---|---|---|---|
| *ACTB* | Beta-actin | NM_001101 | Forward: agcctcgcctttgccga | 174 | 1.97 |
| | | | Reverse: ctggtgcctggggcg | | |
| | | | Probe: F-ccgccgcccgtccacacccgccT-P | | |
| *ALAS1* | Aminolevulinate, delta-, synthase 1 | NM_000688 | LightCycler-h-ALAS Housekeeping Gene Set | 127 | 1.97 |
| | | | Roche, cat. no. 03 302 504 001 | | |
| *ALB* | Albumin | NM_000477 | Forward: tgccctgtgcagaagactatcta | 261 | 1.93 |
| | | | Reverse: cgagctcaacaagtgcagtt | | |
| | | | Probe: F-aagtgacagagtcaccaaatgcTgcac-P | | |
| *B2M* | Beta-2-microglobulin | NM_004048 | Forward: agcgtactccaaagattcaggtt | 306 | 1.91 |
| | | | Reverse: atgatgctgcttacatgtctcgat | | |
| | | | Probe: F-tccatccgacattgaagtTgacttactg-P | | |
| *G6PD* | Glucose-6-phosphate dehydrogenase | NM_000402 | Forward: atcgaccactacctgggcaa | 191 | 1.92 |
| | | | Reverse: ttctgcatcacgtcccgga | | |
| | | | Probe: F-aagatcctgttggcaaatcTcagcacca-P | | |
| *GAPD* | Glyceraldehyde-3-phosphate dehydrogenase | NM_002046 | Forward: gaaggtgaaggtcggagtc | 226 | 1.92 |
| | | | Reverse: gaagatggtgatgggatttc | | |
| | | | Probe: F-caagcttcccgttctcagccT-P | | |
| *HMBS* | Hydroxymethylbilane synthase | NM_000190 | LightCycler-h-PBGD Housekeeping Gene Set | 150 | 1.98 |
| | Alias: Porphobilinogendeaminase (PBGD) | | Roche, cat. no. 03 146 073 001 | | |
| *HPRT1* | Hypoxanthine phosphoribosyltransferase 1 | NM_000194 | LightCycler-h-HPRT Housekeeping Gene Set | 181 | 1.97 |
| | | | Roche, cat. no. 03 261 891 001 | | |

| Gene symbol | Gene name | Accession no. | Primer/Probe sequence [5′→3′][a] | Amplicon size (bp) | PCR efficiency[b] |
|---|---|---|---|---|---|
| *K-ALPHA-1* | K-alpha-1 tubulin, alpha, ubiquitous | NM_006082 | Forward: tggaacccacagtcattgatga | 135 | 1.95 |
| | | | Reverse: tgatctccttgccaatggtgta | | |
| | | | Probe: F-agatgctgccaataacTatgcccgagg-P | | |
| *POLR2A* | Polymerase (RNA) II (DNA-directed) polypeptide A 220 kDa | NM_000937 | Forward: gcaccacgtccaatgacat | 267 | 1.96 |
| | Alias: RNA polymerase II (RPOL2) | | Reverse: gtgcggctgcttccataa | | |
| | | | Probe: F-taccacgtcatctcctttgatggctcctaT-P | | |
| *PPIA* | Peptidylprolyl isomerase A | NM_021130 | Forward: catctgcactgccaagactgag | 326 | 1.91 |
| | Alias: Cyclophilin A (CYPA) | | Reverse: tgcaatccagctaggcatg | | |
| | | | Probe: F-ttcttgctggtcttgccatTcctgga-P | | |
| *RPL13A* | Ribosomal protein L13a | NM_012423 | Forward: cggaccgtgcgaggtat | 114 | 1.92 |
| | | | Reverse: caccatccgctttttcttgtc | | |
| | | | Probe: F-ctgccccacaaaaccaagcgaggccT-P | | |
| *SDHA* | Succinate dehydrogenase complex, subunit A, flavoprotein (Fp) | NM_004168 | Forward: cactggaggaagcacaccc | 78 | 1.92 |
| | | | Reverse: ccttcccagtgccaacgtccacaat | | |
| | | | Probe: F-ccttcccagtgccaacgtccacaaT-P | | |
| *TBP* | TATA box binding protein | NM_003194 | Forward: ttcggagagttctgggattgta | 227 | 1.94 |
| | | | Reverse: tggactgttcttcactcttggc | | |
| | | | Probe: F-ccgtggttcgtggctctcttatcctcaT-P | | |
| *UBC* | Ubiquitin C | NM_021009 | Forward: tcgagaatgtcaaggcaaagatc | 132 | 2.0 |
| | | | Reverse: gagtggactctttctggatgttgta | | |
| | | | Probe: F-tcagacagggtgcgtccatctTccag-P | | |

| Gene symbol | Gene name | Accession no. | Primer/Probe sequence [5′→3′][a] | Amplicon size (bp) | PCR efficiency[b] |
|---|---|---|---|---|---|
| *YWHAZ* | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | NM_003406 | Forward: aagttcttgatccccaatgctt | 196 | 1.95 |
| | Alias: Phospholipase A2 (PLA) | | Reverse: gtctgataggatgtgttggttgc | | |
| | | | Probe: F-tatgcttgttgtgactgatcgacaatcccT-P | | |

[a] *T* TAMRA-labeled, *F* FAM-labeled, *P* phosphate
[b] PCR efficiencies were calculated according to Rasmussen [24]

**Table 2** Candidate reference genes for normalization and the best combination of two genes listed according to their expression stability calculated by the NormFinder program

| Ranking order | Gene | Stability value[a] |
|---|---|---|
| 1 | *HPRT1* | 0.031 |
| 2 | *ALAS1* | 0.044 |
| 3 | *K-ALPHA-1* | 0.055 |
| 4 | *SDHA* | 0.063 |
| 5 | *POLR2A* | 0.080 |
| 6 | *G6PD* | 0.083 |
| 7 | *GAPD* | 0.106 |
| 8 | *TBP* | 0.111 |
| 9 | *PPIA* | 0.115 |
| 10 | *YWHAZ* | 0.124 |
| 11 | *B2M* | 0.132 |
| 12 | *UBC* | 0.149 |
| 13 | *ALB* | 0.205 |
| Best combination of two genes | *HPRT1 + ALAS1* | 0.027 |

[a]High expression stability is indicated by a low stability value as an estimate of the combined intra- and intergroup variation of the individual gene