Mol Biol Rep (2013) 40:2955–2962
DOI 10.1007/s11033-012-2366-5

Reference gene for primary culture of prostate cancer cells

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Received: 12 July 2012 / Accepted: 17 December 2012 / Published online: 27 December 2012

Abstract Selection of reference genes to normalize mRNA levels between samples is critical for gene expression studies because their expression can vary depending on the tissues or cells used and the experimental conditions. We performed ten cell cultures from samples of prostate cancer. Cells were divided into three groups: control (with no transfection protocol), cells transfected with siRNA specific to knockdown the androgen receptor and cells transfected with inespecific siRNAs. After 24 h, mRNA was extracted and gene expression was analyzed by Real-time qPCR. Nine candidates to reference genes for gene expression studies in this model were analyzed (*aminolevulinate, delta-, synthase 1 (ALAS1)*; *beta-actin (ACTB)*; *beta-2-microglobulin (B2M)*; *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*; *hypoxanthine phosphoribosyltransferase 1 (HPRT1)*; *succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA)*; *TATA box binding protein (TBP)*; *ubiquitin C (UBC); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)*). Expression stability was calculated NormFinder algorithm to find the most stable genes. NormFinder calculated *SDHA* as the most stable gene and the gene with the lowest intergroup and intragroup variation, and indicated *GAPDH* and *SDHA* as the best combination of two genes for the purpose of normalization. Androgen receptor mRNA expression was evaluated after normalization by each candidate gene and showed statistical difference in the transfected group compared to control group only when normalized by combination of *GAPDH* and *SDHA*. Based on the algorithm analysis, the combination of *SDHA* and *GAPDH* should be used to normalize target genes mRNA levels in primary culture of prostate cancer cells submitted to transfection with siRNAs.

Keywords Prostate cancer cells · Primary culture · Housekeeping genes · siRNA

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Introduction

Prostate cancer (PCa) is the sixth most common cancer in the world and the most prevalent in men, accounting for 10 % of all types of cancers. In Brazil PCa is the second main cause of cancer death among men [1]. Despite the rising knowledge about the hormonal, nutritional, and environmental context of PCa, several mechanisms concerning the pathogenesis of prostate cancer have to be clarified. PCa is a heterogeneous

disease with variation in clinical aggressiveness, and its behavior could be a direct or an indirect result of gene expression alterations in prostate epithelial cells [2]. However, the molecular events by which PCa progresses from an asymptomatic and non-life-threatening disease to a life-threatening disease are not well understood [2]. Thus, many researchers are using the gene expression profile of prostate tumors to detect alterations related to tumor development [3–5]. The establishment of a gene expression profile for prostate cancer will contribute to patients' prognosis, tumor stratification, classification of insignificant PCa, development of tools for early detection, and identification of therapeutic targets [2].

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a well established, easy to perform technique used in gene expression studies because it allows fast, accurate, and sensitive evaluation of mRNA levels in biological samples [6]. As RT-PCR is a multiple step method, from sample collection to amplification data analysis, there is an inherent variability in its use, which may result in gene expression data distinct from the actual data. Therefore, an appropriate normalization strategy for the quantitative data is necessary to make accurate comparison between samples. Without appropriate normalization, the expression profile of a gene could be erroneously interpreted [7]. Several normalization strategies have been proposed, however, the use of reference genes is the gold standard to normalize mRNA fractions from biological samples [8].

Housekeeping genes (HKGs), also called reference genes or maintenance genes, maintain basic cell metabolic functions and provide support to cell cycle [9]. To be used as a reference gene, a gene should meet the following criteria: stability, non-regulated expression in the analyzed samples, absence of variation under experimental treatment conditions, and similar expression levels to transcript levels of the target gene [10]. Constitutive genes or housekeeping genes meet those criteria and have been used for normalization in many gene expression studies [11].

Even though an inappropriate normalization can result in inadequate quantification of mRNA levels and erroneous conclusions about gene expression profile, the use of reference genes commonly accepted as housekeeping genes without prior validation of this gene under experimental conditions is frequent [12]. Evidence shows that there is not an universal reference gene [6]; therefore, the gene should be chosen and validated considering the tissue and specific experimental conditions applied to the samples [13].

There is no information about a validated housekeeping gene for gene expression studies in primary culture of prostate cancer cells. Most of the previous gene expression studies with prostatic tissues and cell lines have used the following genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [14], *beta-actin* [2, 15, 16], and *beta-2-microglobulin* (*B2M*) [17]. However, studies have shown variation in some of those genes in prostatic tissue [18–20], suggesting that they are inappropriate for normalization in gene expression studies of prostate cells.

The objective of the present study was to identify appropriate reference genes for normalization in gene expression studies using RT-PCR in primary culture of prostate cancer cells submitted to androgen receptor silencing by small interfering RNAs (siRNAs).

Materials and methods

Materials and reagents

Hank's solution, kanamycin sulfate, and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). DMEM high glucose was purchased from LGC Biotecnologia (Cotia, SP, Brazil). Six-well plates were purchased from Nunc (Thermo Fischer Scientific, Roskilde, Denmark). RNAi reagents were obtained from Upstate (Charlottesville, Virginia, USA).

Primary culture of prostate cancer cells

Primary culture was performed from a fragment of tumor collect on the day of surgical procedure (radical prostatectomy or prostatovesiculectomy). Patients were recruited from the Department of Urology of the Hospital de Clínicas de Porto Alegre (HCPA). PCa diagnosis was confirmed by anatomic pathology test. The patients selected were not treated with hormone therapy or chemotherapy, and did not have another type of cancer. All patients provided written consent to participate in the study. The present study was approved by the Research Ethics Committee of the HCPA.

Ten primary cultures were performed. The initial fragment was placed in Hank's solution plus 0.5 mg/mL kanamycin sulfate. The tissue was sectioned in 2 × 2 mm fragments (explants), and the explants were plated in 1 mL of FBS in 6-well plates (approximately six fragments per well). After 24 h, FBS was replaced with 1 mL of culture medium (DMEM high glucose supplemented with 10 % FBS (v/v) and 0.5 mg/mL kanamycin sulfate). The cultures were kept in 5 % CO_2 at 37 °C for approximately 10 days, and the medium was replaced every 48 h. For the purpose of reference genes mRNA expression analysis in cells submitted to the transfection protocol, the cells were divided into two groups: a control group (non-transfected cells) and a transfected group (with androgen receptor siRNA or non-specific siRNA).

Androgen receptor silencing

Androgen receptor siRNAs and negative controls (non-specific siRNAs) were obtained from Upstate (SMART-pool®) (Charlottesville, Virginia, USA). The transfection reagent used was siIMPORTER™ Transfection Reagent (Upstate, Charlottesville, Virginia, USA). All transfections were done according to the manufacturer's protocol.

Total RNA extraction

Total RNA was extracted using the reagent Trizol (Invitrogen, Carlsbad, CA, USA). Cells were lysed directly in wells containing 1 mL of Trizol per 10 cm^2. The extraction followed the manufacturer's protocol. Total RNA was quantified by the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 and 280 nm wavelengths.

Real-time reverse transcription polymerase chain reaction

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with Oligo $(DT)_{12–18}$ primer, using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

RT-PCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). cDNA samples were amplified on StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a total volume of 12.5 μL [6.25 μL of SuperMix, 1 μL of 50 μmol/L Rox dye, 0.1 μL of each primer (10 μmol/L forward and 10 μmol/L reverse)], 1 μL of 100× diluted sample, and 4.05 μL of water. Quantification of amplified samples was performed based on amplification of a standard curve (serial dilution of 4 ng/μL standard cDNA).

Primer design

Primers were designed with PrimeTime qPCR Assay Entry (IDT, Integrated DNA Technologies, Coralville, Iowa, USA), except for the androgen receptor primer, which was designed with Primer3 [21] and synthesized by IDT (Table 1).

The genes included in the study were: *aminolevulinate, delta-, synthase 1 (ALAS1); beta-actin (ACTB); beta-2-microglobulin (B2M); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); hypoxanthine phosphoribosyltransferase 1 (HPRT1); succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA); TATA box binding protein (TBP); ubiquitin C (UBC); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)*. All these genes have been previously evaluated in different tissues and sample types [12, 22].

Statistical analysis

Analysis of quantification data was done with the NormFinder [23] algorithm, which is a Visual Basic application for Microsoft Excel. Data were subdivided into control group (control samples, non-transfected cells) and transfected group (androgen receptor siRNA or negative control). The data of androgen receptor mRNA quantification were normalized by each reference gene data and also by a combination of two genes. The data of androgen receptor expression of the transfected group of each culture was relativized to the control group data and analyzed using Kruskal–Wallis test (Dunn's multiple comparisons post hoc test) in the SPSS Statistics 20.0 (SPSS Inc).

Results

To identify the best reference gene for sample normalization in gene expression studies in primary culture of prostate cancer cells, we amplified nine genes commonly used as control genes (*ALAS1, ACTB, B2M, GAPDH, HPRT1, SDHA, TBP, UBC,* and *YWHAZ*). The samples were diluted 100× and nevertheless the sample CT values for the ubiquitin C gene extrapolated the highest concentration point of the standard curve; thus it was excluded from the analysis. Mean C_T values, standard deviation (SD), coefficient of variation (CV), and maximum fold change (MFC-ratio of the maximum and minimum values) are showed in Table 2, in ascending order of CV. These values represent the first analysis of dispersion data, and could suggest the least variable gene. However, as demonstrated by the minimum and maximum values, none of the genes had a constant expression, which may indicate that the dispersion data is not sufficient to identify an adequate reference gene in primary culture of prostate cancer cells transfected with siRNA.

We proceed the analysis using NormFinder [23] for quantification data (in ng). This algorithm directly estimates the variation in the expression of each candidate gene, considering systematic differences between sample subgroups [23]. The expression stability of a candidate gene is indicated by its stability value. NormFinder demonstrated that *SDHA* was the most stable gene, with the lowest stability value (Fig. 1a), also showing the smallest intergroup variation (Fig. 1b). The algorithm also suggested the best combination of the two most stable genes to compensate the fluctuation of experimental data in gene expression variations in response to a treatment [24]. This

Table 1 Primers sequence, NCBI reference sequence, cell function and product length for housekeeping genes and target genes amplification by qualitative RT-PCR

Gene symbol	Name	NCBI refseq	Strand	Sequence (5'–3')	Product length (bp)	Cell function
ACTB	Beta-actin	NM_001101.3	Forward	CTGGAACGGTGAAGGTGACA	140	Cytoskeletal structural protein
			Reverse	AAGGGACTTCCTGTAACAATGCA		
ALAS1	Aminolevulinate, delta-, synthase 1	NM_199166.1	Forward	AGTGTGAAAACCGATGGAGG	140	Heme synthesis
			Reverse	CGATCATACTGAAAAGTGGAAACAG		
B2M	Beta-2-microglobulin	NM_004048.2	Forward	CTATCCAGCGTACTCCAAAG	165	Major Histocompatibility Complex
			Reverse	ACAAGTCTGAATGCTCCACT		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.3	Forward	CTTTGTCAAGCTCATTTCCTGG	133	Glycolytic enzyme
			Reverse	TCTTCCTCTTGTGCTCTTGC		
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194.2	Forward	AGATGGTCAAGGTCGCAAG	128	Metabolic salvage of purines
			Reverse	GTATTCATTATAGTCAAGGGCATATCC		
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_004168.2	Forward	TGGTTGTCTTTGGTCGGG	85	Electron transporter in TCA cycle and respiratory chain
			Reverse	GCGTTTGGTTTAATTGGAGGG		
TBP	TATA box binding protein	NM_003194.4	Forward	GGGTTTTCCAGCTAAGTTCTTG	150	Regulation of transcription
			Reverse	CTGTAGATTAAACCAGGAAATAAC		
UBC	Ubiquitin C	NM_021009.5	Forward	GCCTTAGAACCCCAGTATCAG	74	Protein degradation
			Reverse	AAGAAAACCAGTGCCCTAGAG		
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_001135699.1	Forward	CAACACATCCTATCAGACTGGG	133	Signal transduction
			Reverse	AATGTATCAAGTTCAGCAATGGC		
AR	Androgen receptor	NM_000044.2	Forward	CTACTCCGGACCTTACGGGGACATGCG	427	Steroid-hormone activated transcription factor
			Reverse	GGGCTGACATTCATAGCCTTCAATGTGTGAC		

bp base pair

Table 2 Dispersion data of raw CT values for eight candidate control genes

Gene	Mean	SD[a]	CV[b] (%)	Minimum	Maximum	MFC[c]
ALAS1	29.83	2.25	7.55	27.36	34.59	1.26
YWHAZ	24.77	2.05	8.26	20.66	29.62	1.43
HPRT1	31. 40	2.65	8.43	27.21	35.96	1.31
SDHA	29.43	2.50	8.49	26.35	35.19	1.34
B2M	21.15	1.86	8.78	18.82	25.09	1.33
GAPDH	21.97	2.13	9.68	19.62	26.45	1.35
ACTB	22.35	2.39	10.71	18.78	27.12	1.44
TBP	30.33	3.39	11.17	24.99	36.86	1.47

[a] *SD* standard deviation

[b] *CV* coefficient of variation (ratio of SD and mean)

[c] *MFC* maximum fold change (ratio of maximum and minimum values)

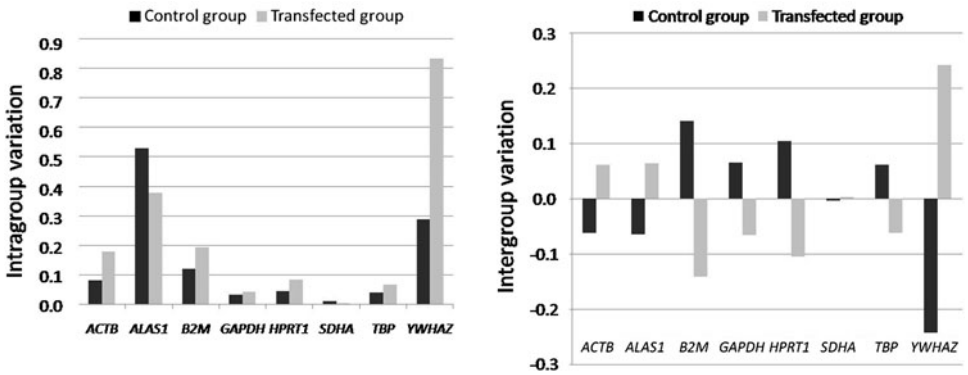

Fig. 1 Intra- (**a**) and intergroup variation (**b**) of eight reference genes in samples of primary culture of prostate cancer cells, as determined by NormFinder, showing *SDHA* as the gene with the smallest (most stability) and *YWHAZ* with the highest variation (less stability). *Control group* cells without transfection protocol, *transfected group* cells transfected with siRNA (against AR or inespecific control)

Table 3 Stability values of eight candidate reference genes and of the combination of two genes for normalization of RT-qPCR in primary culture of prostate cancer cells, as calculated by NormFinder

Ranking order	Gene name	Stability value
1	*SDHA*	0.035
2	*SDHA* and *GAPDH*	0.070
3	*GAPDH*	0.127
4	*TBP*	0.132
5	*ACTB*	0.161
6	*HPRT1*	0.171
7	*BMG*	0.220
8	*ALAS1*	0.237
9	*YWHAZ*	0.297

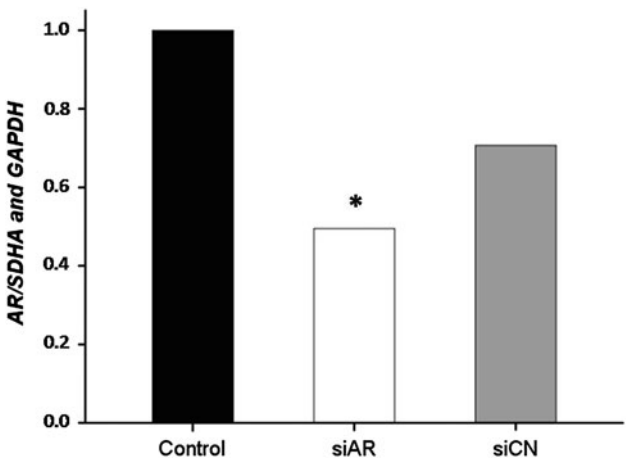

Fig. 2 *AR* relative gene expression in primary culture of prostate cancer cells with normalization to the combination of two reference genes (*SDHA* and *GAPDH*). The bars represent the median of the groups relativized to control. Control-control group (non-transfected); siAR group-transfected with specific RNA to androgen receptor; siNC group-transfected with non-specific siRNA (negative control). *Statistically significant difference in gene expression between siAR and control group, $P < 0.05$ (Kruskal–Wallis test, Dunn's post hoc test

algorithm considered *SDHA* and *GAPDH* as the best combination of two genes. Table 3 shows the stability values of candidate genes, and also the stability value of the combination *SDHA* and *GAPDH*.

One of the main target genes in our studies is the androgen receptor (*AR*) gene. We evaluate the expression of the *AR* mRNA in control and transfected groups. The quantified data from amplification of the *AR* mRNA were normalized by each of the eight candidate reference genes and also by the combination of the two genes suggested by NormFinder. The ratio *AR/control gene* was relativized to the control group value in each culture. Figure 2 shows the *AR* gene expression in the transfected group (androgen receptor siRNA or negative control) compared with the control group for the combination of *GAPDH* and *SDHA* genes. Only when normalized by the combination of *SDHA* and *GAPDH* the *AR* mRNA expression in the siAR group showed statistical difference between the control and negative control groups. We also accessed the *PSA* mRNA expression in samples with 48 h of transfection, and, as

expected, the quantity of *PSA* mRNA was lower in cells transfected with siRNA against the AR (not shown).

Discussion

According to the current consensus on the use of reference genes to accurately quantify gene expression, there is not one single gene that is a "real" universal housekeeping gene. Different approaches have been proposed to identify gene expression stability and indicate the best control genes under various experimental conditions and using different cell types, such as the algorithms geNorm and NormFinder [8, 23, 25–27]. However, because these strategies are based on different algorithms and analytical procedures, each software produces a different set of top ranked housekeeping genes [26], and the recommendation is the use of only one of the tools available to choose stable housekeeping genes [28]. We decided to use NormFinder to analyze our data of prostate cancer cells.

geNorm ranks the genes according to the pairwise variation with all other control genes and defines a measure of gene stability (*M* value) of a particular gene compared with all other genes [27]. However, this software analyzes all samples, regardless of the differences between control samples and experimental samples. NormFinder, on its turn, provides the overall expression variation and also the variation across subgroups of samples, top ranking the candidates with minimal estimated intra- and intergroup variation [23]. According to Andersen et al. [23], discrepancies in the results of these two strategies are caused by the differences between the approaches. As NormFinder considers intra- and intergroup variation in order to top rank the most stable genes, we suggest that *SDHA* may be the best choice for normalization of mRNA levels amplified by quantitative RT-PCR in primary culture of prostate cancer cells, since these cultures are performed to evaluate the influence of a given treatment on gene expression, thus generating subgroups of samples.

Algorithms like geNorm indicated the optimal number of reference genes to generate an accurate NF for normalization, while NormFinder only shows the best combination of two genes. In spite of the recommendation to use more than one reference gene for an accurate normalization in animal and vegetal species [26, 27, 29], it may not be feasible when few target genes are being studied or when there is limited amount of RNA available [6, 23]. Cell cultures from a limited tissue sample or a biopsy sample are examples of such conditions. Andersen et al. [23] also showed that the normalization is not necessarily improved by the use of a normalization factor (NF), generated by the use of two or more control genes. The use of a NF is recommended only when the candidate reference genes show significant variation. When this is the case, those genes with opposite-directed intergroup variation should be selected to provide accurate normalization.

In order to evaluate the impact of the use of one or two reference genes on our model, we amplified the androgen receptor mRNA, which was silenced in the cultures (verified by the absence of the AR protein by western blot analysis). We found a statistical difference between the siAR group compared to control group only after normalization by the combination of *SDHA* and *GAPDH* (Fig. 2). However, all cultures showed decreased *AR* mRNA levels in the siAR group when normalized by any candidate gene, as expected, despite the lack of statistical significance (when normalized by SDHA alone, $P = 0.061$).

Some studies have reported the evaluation of the best reference gene to be used in prostatic tissues, but none of them has performed primary culture of prostate cancer cells [11, 12, 22, 30]. Also, many studies accessing gene expression profiles in prostate cancer samples have used only one gene to normalized the target gene expression (*ACTB, GAPDH, TBP, HPRT1*, and others) without a previous evaluation of their stability [22]. Nevertheless, the studies conducted to identify the adequate control gene for prostate samples used different types of samples, such as commercially available cDNA [11], normal, prostatic hyperplasia, and prostatic tumor tissues [12], paired malignant and nonmalignant prostatic tissue [22], and LNCaP cells [30]. Therefore, distinct results were found: *RNA polymerase II* [11], *HPRT1* [12], *HPRT1* alone or the combination of *HPRT1* and *ALAS1*, or *HPRT1*, *ALAS1* and *K-ALPHA-1* [22], *and ribosomal highly-basic 23 kDa protein*, *RPL13A* [30].

We found *SDHA* alone or in combination with *GAPDH* as the most appropriate combination of two genes for normalization in our model using NormFinder. The relative expression of AR shown a decrease in the group siAR, as expected, but only when there was normalization by the combination of *SDHA* and *GAPDH* there was a statistical significant difference. In spite of some studies do not recommend the use of *GAPDH* as a reference gene because it is involved in a direct pathway of carbohydrate metabolism, which may be altered in some cancers [31, 32], our results suggests that when only cancer cells are being analyzed, *GAPDH* could shown a satisfactory stability. In fact, many studies have being done using *GAPDH*, besides other genes like *β-actin*, *TBP* and *HPRT*, in samples of prostate cancer [33–36].

In conclusion, the results of the present study suggest that *SDHA* or the combination of *SDHA* and GAPDH should be used for normalization purpose in gene expression analysis in primary culture of prostate cancer cells submitted to siRNA transfection procedure. In addition, we recommend that a preliminary evaluation of the expression

stability of several candidate control genes is performed in order to avoid inaccurate normalization and unnecessary expenditures.

Acknowledgments This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant number 474847/2008-0), the Fundo de Incentivo à Pesquisa (FIPE, Grant number 08-172) of Hospital de Clínicas de Porto Alegre, and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

1. INCA (2011) Estimate/2012-Cancer incidence in Brazil. INCA. http://www.inca.gov.br/estimativa/2012/. Accessed 15 Sept 2011
2. Luo JH, Yu YP, Cieply K, Lin F, Deflavia P, Dhir R, Finkelstein S, Michalopoulos G, Becich M (2002) Gene expression analysis of prostate cancers. Mol Carcinog 33(1):25–35. doi:10.1002/mc.10018
3. Kristiansen G, Pilarsky C, Wissmann C, Kaiser S, Bruemmendorf T, Roepcke S, Dahl E, Hinzmann B, Specht T, Pervan J, Stephan C, Loening S, Dietel M, Rosenthal A (2005) Expression profiling of microdissected matched prostate cancer samples reveals CD166/MEMD and CD24 as new prognostic markers for patient survival. J Pathol 205(3):359–376. doi:10.1002/path.1676
4. Nelson PS (2004) Predicting prostate cancer behavior using transcript profiles. J Urol 172(5 Pt 2):S28–S32 discussion S33
5. Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C, Tamayo P, Renshaw AA, D'Amico AV, Richie JP, Lander ES, Loda M, Kantoff PW, Golub TR, Sellers WR (2002) Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 1(2):203–209
6. Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6(4):279–284. doi:10.1038/sj.gene.6364190
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(2):293–300
8. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A (2007) Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898. doi:10.1371/journal.pone.0000898
9. Khimani AH, Mhashilkar AM, Mikulskis A, O'Malley M, Liao J, Golenko EE, Mayer P, Chada S, Killian JB, Lott ST (2005) Housekeeping genes in cancer: normalization of array data. Biotechniques 38(5):739–745
10. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29(1):23–39
11. 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(4):856–862
12. 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(1):154–159. doi:10.1038/labinvest.3700208
13. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR–a perspective. J Mol Endocrinol 34(3):597–601. doi:10.1677/jme.1.01755
14. Rosner IL, Ravindranath L, Furusato B, Chen Y, Gao C, Cullen J, Sesterhenn IA, McLeod DG, Srivastava S, Petrovics G (2007) Higher tumor to benign ratio of the androgen receptor mRNA expression associates with prostate cancer progression after radical prostatectomy. Urology 70(6):1225–1229. doi:10.1016/j.urology.2007.09.010
15. Kinoshita M, Nakagawa T, Shimizu A, Katsuoka Y (2005) Differently regulated androgen receptor transcriptional complex in prostate cancer compared with normal prostate. Int J Urol 12(4):390–397. doi:10.1111/j.1442-2042.2005.01093.x
16. Stephan C, Yousef GM, Scorilas A, Jung K, Jung M, Kristiansen G, Hauptmann S, Kishi T, Nakamura T, Loening SA, Diamandis EP (2004) Hepsin is highly over expressed in and a new candidate for a prognostic indicator in prostate cancer. J Urol 171(1):187–191. doi:10.1097/01.ju.0000101622.74236.94
17. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP (1995) Mutation of the androgen receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332(21):1393–1398
18. Gross M, Top I, Laux I, Katz J, Curran J, Tindell C, Agus D (2007) Beta-2-microglobulin is an androgen-regulated secreted protein elevated in serum of patients with advanced prostate cancer. Clin Cancer Res 13(7):1979–1986. doi:10.1158/1078-0432.CCR-06-1156
19. Harada N, Yasunaga R, Higashimura Y, Yamaji R, Fujimoto K, Moss J, Inui H, Nakano Y (2007) Glyceraldehyde-3-phosphate dehydrogenase enhances transcriptional activity of androgen receptor in prostate cancer cells. J Biol Chem 282(31):22651–22661. doi:10.1074/jbc.M610724200
20. Shi C, Zhu Y, Su Y, Chung LW, Cheng T (2009) Beta2-microglobulin: emerging as a promising cancer therapeutic target. Drug Discov Today 14(1–2):25–30. doi:10.1016/j.drudis.2008.11.001
21. Rozen S, Skaletsky H (2000) Primer3 on the www for general users and for biologist programmers. Methods Mol Biol 132:365–386
22. Ohl F, Jung M, Xu C, Stephan C, Rabien A, Burkhardt M, Nitsche A, Kristiansen G, Loening SA, Radonic A, Jung K (2005) Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization? J Mol Med 83(12):1014–1024. doi:10.1007/s00109-005-0703-z
23. 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(15):5245–5250. doi:10.1158/0008-5472.CAN-04-0496
24. Santos AR, Duarte CB (2008) Validation of internal control genes for expression studies: effects of the neurotrophin BDNF on hippocampal neurons. J Neurosci Res 86(16):3684–3692. doi:10.1002/jnr.21796
25. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: bestkeeper–excel-based tool using pair-wise correlations. Biotechnol Lett 26(6):509–515
26. Tong Z, Gao Z, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:71. doi:10.1186/1471-2199-10-71
27. 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(7): RESEARCH0034
28. Wan Q, Whang I, Choi CY, Lee JS, Lee J (2011) Validation of housekeeping genes as internal controls for studying biomarkers of endocrine-disrupting chemicals in disk abalone by real-time PCR. Comp Biochem Physiol C 153(3):259–268. doi:10.1016/j.cbpc.2010.11.009
29. Lisowski P, Pierzchala M, Goscik J, Pareek CS, Zwierzchowski L (2008) Evaluation of reference genes for studies of gene

expression in the bovine liver, kidney, pituitary, and thyroid. J Appl Genet 49(4):367–372. doi:47010.1007/BF03195635
30. Mogal A, Abdulkadir SA (2006) Effects of histone deacetylase inhibitor (HDACi); trichostatin-A (TSA) on the expression of housekeeping genes. Mol Cell Probes 20(2):81–86
31. Blanquicett C, Johnson MR, Heslin M, Diasio RB (2002) Housekeeping gene variability in normal and carcinomatous colorectal and liver tissues: applications in pharmacogenomic gene expression studies. Anal Biochem 303(2):209–214. doi:10.1006/abio.2001.5570
32. Unwin RD, Craven RA, Harnden P, Hanrahan S, Totty N, Knowles M, Eardley I, Selby PJ, Banks RE (2003) Proteomic changes in renal cancer and co-ordinate demonstration of both the glycolytic and mitochondrial aspects of the Warburg effect. Proteomics 3(8):1620–1632. doi:10.1002/pmic.200300464
33. Braun M, Menon R, Nikolov P, Kirsten R, Petersen K, Schilling D, Schott C, Gundisch S, Fend F, Becker KF, Perner S (2011) The HOPE fixation technique–a promising alternative to common prostate cancer biobanking approaches. BMC Cancer 11:511. doi:10.1186/1471-2407-11-511
34. Floriano-Sanchez E, Cardenas-Rodriguez N, Castro-Marin M, Alvarez-Grave P, Lara-Padilla E (2009) DD3(PCA3) gene expression in cancer and prostatic hyperplasia. Clin Invest Med 32(6):E258
35. Mavridis K, Avgeris M, Koutalellis G, Stravodimos K, Scorilas A (2010) Expression analysis and study of the KLK15 mRNA splice variants in prostate cancer and benign prostatic hyperplasia. Cancer Sci 101(3):693–699. doi:10.1111/j.1349-7006.2009.01450.x
36. Mori R, Wang Q, Danenberg KD, Pinski JK, Danenberg PV (2008) Both beta-actin and GAPDH are useful reference genes for normalization of quantitative RT-PCR in human FFPE tissue samples of prostate cancer. Prostate 68(14):1555–1560. doi:10.1002/pros.20815