

# Housekeeping Gene Sets Facilitate the Search for a Suitable Reference Gene for Relative Quantification



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**We describe the search for a suitable housekeeping gene for relative quantification of prostate-specific antigen (PSA) mRNA-bearing cells in blood using the LightCycler Instrument. The practicability of the two LightCycler Housekeeping Gene Sets, h- $\beta$ 2M and h-PBGD, is examined for this purpose. h-PBGD complies with the requirements for a reference gene to quantify PSA mRNA using this approach.**

## Introduction

In several cancer studies, the detection of disseminated tumor cells in peripheral blood as a sign of tumor spread and micrometastasis (minimal residual diseases) has been discussed as a possible indicator for early diagnosis, as well as therapy selection and control [1]. The detection of these cells requires first a cancer cell-specific or tissue-specific molecular marker and second the application of sensitive polymerase chain reaction (PCR)-based detection systems with high analytical reliability. In prostate carcinoma, the determination of the prostate-specific antigen (PSA) mRNA as an organ-specific gene in disseminated tumor cells has been recommended and investigated with different results [2].

The aim of our research is to examine the occurrence of prostatic cells in blood from subjects suffering from benign and malignant prostatic diseases, in relation to the conventional marker total PSA and its different molecular forms in serum. We decided – in the absence of a PSA mRNA standard – to perform relative quantification of the PSA mRNA-bearing cells with an endogenous reference gene (housekeeping gene).

A housekeeping gene used for relative mRNA quantification should generally fulfil the following criteria [3]:

- ➔ It should exhibit constitutively, non-regulated expression in the sample types investigated.
- ➔ The detection should be RNA-specific; a pseudo-gene- and DNA-free amplification should be realized by stringent primer design.
- ➔ It should be expressed in a range similar to the target gene in the samples to be analyzed.

- ➔ It should be amplified with a PCR efficiency identical to the target gene and external standard.

Here, we report on the search for a suitable housekeeping gene for the relative quantification of the target gene (PSA) in blood using the LightCycler technology. We tested the first two available LightCycler Housekeeping Gene Sets for human  $\beta_2$ -microglobulin ( $\beta$ 2M) and human porphobilinogen deaminase (PBGD). Our goal was to clarify whether one of these two housekeeping genes could be used for our application, taking into account, in particular, points 3 and 4 mentioned above.

We generated standard curves for both genes (according to the instruction sheets), and examined the expression levels of  $\beta$ 2M and PBGD in samples of peripheral blood and cells of the transformed prostate cancer cell line LNCaP. These cells produce PSA and are generally used as an *in vitro* model for prostate-cancer studies. The detection of one LNCaP cell among  $10^6$  non-PSA producing cells is considered the gold standard for a sensitive method of PSA mRNA detection. Therefore, the detection limit was estimated in this respect to characterize the quantification of low numbers of circulating prostate-cancer cells in blood with real-time PCR of the LightCycler Instrument.

## Materials and Methods

### Sample material

- ➔ RNA standards: The  $\beta$ 2M and the PBGD standards were applied as ready-to-use RNA solutions from the housekeeping gene sets mentioned above. The PBGD standard RNA was additionally diluted to  $1 \times 10^2$  and

$5 \times 10^1$  copies with 12 ng/ $\mu$ l MS2 RNA (Roche Applied Science).

→ Blood samples: 5 ml fresh-drawn K-EDTA blood was immediately transferred into plastic tubes containing 10 ml RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Applied Science), mixed, and stored at  $-20^\circ\text{C}$  until RNA isolation was to be performed.

### LNCaP cells and serial dilutions

LNCaP cells (ATCC Rockville, MD, USA) grown in RPMI-1640 medium (Life Technologies, Karlsruhe, Germany) with 10% fetal-calf serum and antibiotics were harvested after 80% confluence, washed, and counted. A definitive cell number was used for serial dilutions in blood samples of a female blood donor without PSA mRNA. A cell suspension of 2,500 LNCaP cells/100  $\mu$ l phosphate-buffered saline (PBS) was added to 5 ml K-EDTA blood mixed with 10 ml RNA/DNA Stabilization Reagent. The mix was diluted with stabilized blood up to 12 LNCaP cells/5 ml blood.

### Total RNA isolation

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science). The stabilized blood samples were directly applied on the filter tube and were isolated twice. Two filter tubes were loaded with 750  $\mu$ l stabilized blood twice. After binding/washing steps, RNA was removed from the first tube with 60  $\mu$ l of elution buffer, and used for the second filter tube as elution solution. Thus, RNA was isolated from a total 1 ml whole blood. The isolation was not quantitative but the

yield of RNA was 30–50% higher than that achieved by double isolation in one tube.

### cDNA synthesis

The 1<sup>st</sup> Strand cDNA Synthesis Kit (Roche Applied Science) was used (hexamer priming). For the  $\beta$ 2M and PBGD RNA standards, 5  $\mu$ l RNA was reverse transcribed. For the blood samples, the maximum volumes of RNA samples were used, but the RNA amount did not exceed 1  $\mu$ g RNA.

### LightCycler PCR

Real-time PCRs for  $\beta$ 2M, PBGD, and PSA were performed with the LightCycler FastStart DNA Master Hybridization Probes and the LightCycler Instrument. In general, a total volume of 20  $\mu$ l included 2  $\mu$ l cDNA as template. The reaction conditions (according to the instruction sheets) were the same for both genes, as well as for the PSA cDNA amplification. Final concentrations for PSA-PCR: 4 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{M}$  primers, and 0.2  $\mu\text{M}$  Hybridization Probes. The sequences (5' to 3') of primers were: GAACCA-GAGGAGTTCTT (forward) and CCCAGAATCACCCGAG (reverse), and of the hybridization probes: CTTGCGCACACACGTCATTGGAA-Fluorescein and LightCycler Red640-TAACATGGAGGTCCACACACTGAA-P. Data analyses were performed with the Second Derivative Maximum (SDM).

### Results and Applications

The results of this study, which tested the first two available housekeeping gene sets regarding their usefulness

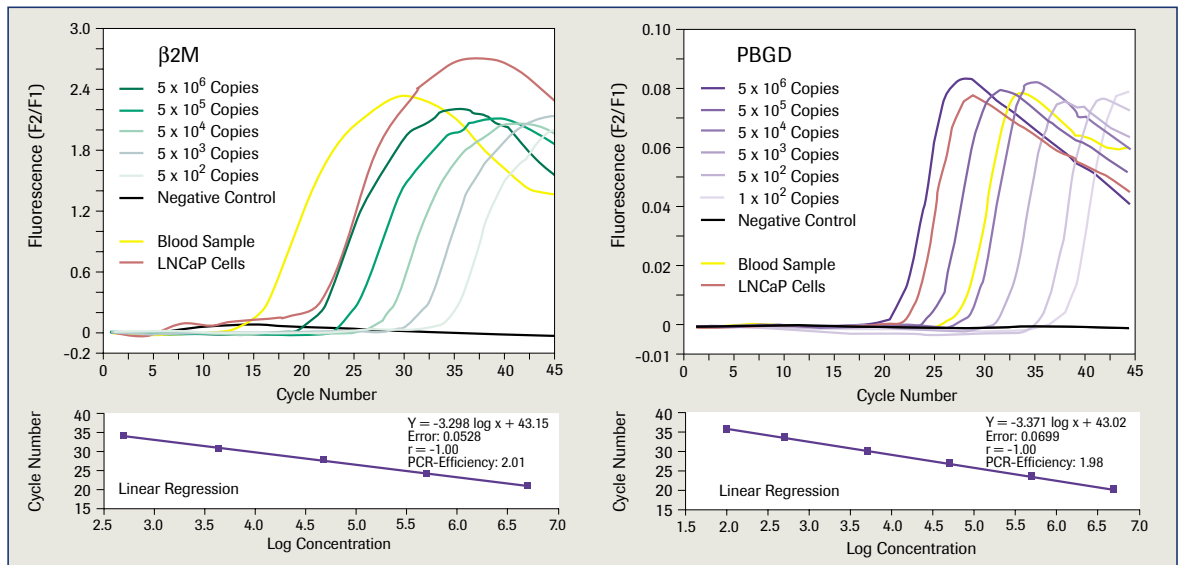


Figure 1: Real-time amplification plots (upper part) of reverse transcribed ready-to-use RNA standard solutions of the h- $\beta$ 2M and h-PBGD housekeeping gene sets. The standard curve for h-PBGD was extended up to 100 copies. Expression levels in a blood sample and in  $1 \times 10^5$  LNCaP cells. Data for  $\beta$ 2M were analyzed with LightCycler Software version 3.0; PBGD was analyzed with version 3.5. Linear regression line (lower part) showing the linearity between cycle number and starting RNA quantity calculated with LightCycler Software (PCR efficiency =  $10^{-1/\text{slope}}$ ).

for the relative quantification of prostate cells in blood, are shown in Figures 1 and 2. Both housekeeping gene sets are easy to handle. Amplification can be performed with 2 µl cDNA, instead of the recommended 5 µl, with equal amplification efficiency (Figure 1).

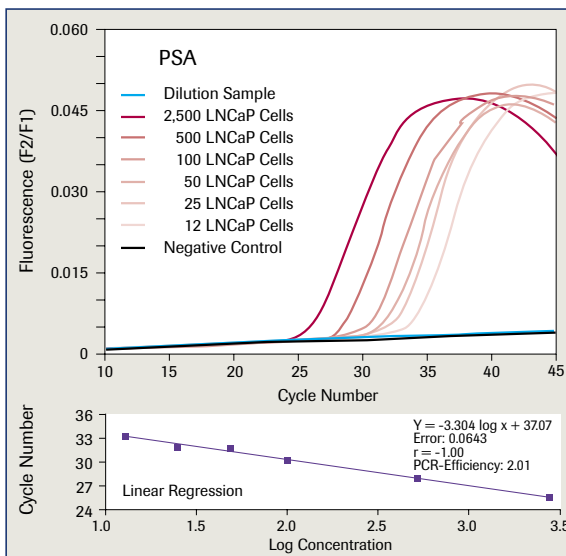
In blood samples and LNCaP cells, β2M mRNA was highly expressed (Figure 1) with copy numbers outside the standard curve. In contrast, the PBGD gene, a low-abundance class housekeeping gene, was moderately expressed, and was in the dynamic range of the standard curve. From this point of view, the PBGD gene, but not the β2M gene, is a suitable housekeeping gene for our sample material and can be used as a reference gene for the normalization of our target gene.

Due to the expected low number of circulating prostatic cells in blood and consequently low PSA mRNA levels [4], we extended the PBGD mRNA standard curve up to 50 copies by dilution as described above. The data analysis with the SDM method recorded amplifications up to 100 copies (crossing point at cycle 36) whereas the Fit Point analysis allowed detection of up to 50 copies (crossing point at cycle 39). The same PCR efficiency for PBGD was achieved in a similar range with diluted cDNA from blood samples (data not shown).

The PCR efficiency of the target gene PSA (Figure 2) was comparable to that achieved by the housekeeping gene in the blood samples, and by the external PBGD mRNA standards. All three efficiencies are within the generally accepted variance limit of 0.05 (between the target and the reference gene), and guarantee a reliable quantification. Thus, the PBGD gene complies with both the above-mentioned requirements (3 and 4). It can be considered a suitable housekeeping gene for our purposes. The PSA mRNA concentration in each blood sample can be expressed as the PSA/PBGD ratio.

In addition, we were able to show (Figure 2) that the detected PSA mRNA in 12 LNCaP cells (in 5 ml stabilized blood) corresponds to one LNCaP cell in a background of 10<sup>6</sup> mononuclear blood cells. It proves the high analytical sensitivity of the described procedure to quantify PSA mRNA using two-step RT-PCR with the LightCycler Instrument.

In conclusion, our studies showed that the LightCycler h-PBGD Housekeeping Gene Set is a useful external standard for the relative quantification of a low-copy gene (e.g., for PSA mRNA-bearing cells in blood). Recently, three new sets of housekeeping genes with different expression levels have been introduced for broad



**Figure 2: Real-time amplification plots (upper part) of reverse-transcribed PSA mRNA from serial dilutions of LNCaP cells in 5 ml stabilized blood samples. Data were analyzed with LightCycler Software version 3.5. The linear regression line (lower part) shows the linearity between cycle number and starting quantity of RNA and is calculated with the LightCycler Software (PCR efficiency = 10<sup>-1/slope</sup>).**

applications in research studies using the LightCycler Instrument (human 5-aminolevulinate synthase h-ALAS, human glucose-6-phosphate dehydrogenase h-G6PDH, and human hypoxanthine phosphoribosyl transferase h-HPRT). These sets greatly facilitate the search for an appropriate housekeeping gene for the relative quantification of a gene of interest.

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## References

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Product	Pack Size	Cat. No.
<b>LightCycler – h-G6PDH Housekeeping Gene Set</b>	1 set (96 reactions)	3 261 883
<b>LightCycler – h-HPRT Housekeeping Gene Set</b>	1 set (96 reactions)	3 261 891
<b>LightCycler – h-β2M Housekeeping Gene Set</b>	1 set (96 reactions)	3 146 081
<b>LightCycler – h-PBGD Housekeeping Gene Set</b>	1 set (96 reactions)	3 146 073
<b>LightCycler – h-ALAS Housekeeping Gene Set</b>	1 set (96 reactions)	3 302 504
<b>LightCycler – h-Housekeeping Gene Selection Set</b>	1 set (5 x 16 reactions)	3 310 159

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