Flexibility in reference gene selection for reliable gene expression analysis

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This application note focuses on choosing reference genes for gene expression analysis. The robustness, accuracy, and sensitivity of the new QuantiNova Probe PCR Kit are investigated in terms of duplex real-time PCR of differently expressed genes. The kit supports flexible reference gene choice, allowing multiple combinations of reference and target genes.

Introduction

In gene expression analysis, it is essential to have an accurate means of checking for errors from sample to sample. A widely used method is relative quantification, which involves determining the ratio between the amounts of the target gene and a reference gene. This ratio can then be compared across the various samples (1).

Reference genes for gene expression analysis are generally endogenous: the target and reference gene are amplified from the same sample, either separately via singleplex real-time PCR or in the same reaction via duplex real-time PCR. The normalized value is determined for each sample and can be used to compare the differential expression of a gene in different tissues or to compare gene expression between siRNA-transfected cells and untransfected cells. When gene expression levels are compared between samples, the expression level of the target is referred to as being, for example, 100-fold higher in stimulated cells than in unstimulated cells.

The expression levels of the reference gene need to be constant among different types of cell or tissue and under different experimental conditions. The expression of some reference genes may vary considerably in certain samples, and this might lead to erroneous results (2). It is therefore crucial to extensively validate reference genes for every experimental model (3). Flexibility in the choice of reference gene is also essential to enable the best choice of experimental setup.

The reference and target genes should ideally be analyzed in a duplex reaction to eliminate well-to-well variability. A prerequisite for reliable

duplex reaction is that the used real-time PCR master mix allows reliable amplification and detection of both target genes, even though they may vary greatly in their abundance. Simultaneous amplification of two highly differently expressed genes can be challenging. A highly abundant gene can out-compete a less abundant gene, leading to a bias in the results.

In this study, the QuantiNova Probe PCR kit was investigated in terms of its robustness, accuracy, and sensitivity for duplex real-time PCR, specifically with regard to using the kit in gene expression analysis. Furthermore, the support for flexibility in reference gene choice was assessed. In both cases, the QuantiNova Probe PCR Kit performed excellently, proving highly suitable for gene expression analysis workflows.

Materials and methods

Ten-fold dilutions of leukocyte cDNA were used for the comparison of duplex and singleplex real-time PCR. The assays were set up using the QuantiNova Probe PCR Kit and TaqMan® Assays for the genes of interest, GAPD and TNF. The assays were run in triplicate on a Bio-Rad® CFX96™ thermal cycler.

To confirm the flexibility in the choice of reference gene, five target genes (ERBB2, MDM2, EGFR, CCND1, and MYC) were co-amplified with each of five reference genes (PPIA, GAPD, ACTB, UBC, and 18S RRN) in 10 ng cDNA from HeLa cells. Singleplex and duplex real-time PCR assays were performed using the QuantiNova Probe PCR Kit on a Rotor-Gene Q thermal cycler.

Results

The QuantiNova Probe PCR Kit enables robust duplex real-time PCR and provides highly accurate duplex results for the two differently expressed genes, GAPD and TNF (Figure 1).

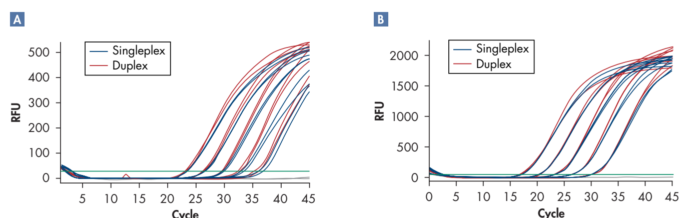

Figure 1. Comparable results in duplex and singleplex PCR. Duplex and singleplex real-time PCR were performed using the QuantiNova Probe PCR Kit and TaqMan Assays on a Bio-Rad CFX96 thermal cycler to amplify the GAPD and TNF transcripts. Ten-fold dilutions of leukocyte cDNA (from 100 ng to 10 pg) were used as the templates and the reactions were run in triplicate. **A** Overlay of the TNF amplification curves for the singleplex and duplex reactions. **B** Overlay of the GAPD amplification curves for the singleplex and duplex reactions. The plots demonstrate the comparability and reliability of the results for singleplex and duplex amplification using the QuantiNova Probe PCR Kit.

The sensitivity and efficiency of the singleplex and duplex assays are similar, demonstrating that the QuantiNova Probe PCR Kit enables reliable and unbiased duplex real-time PCR.

Furthermore, highly consistent results are obtained for various combinations of reference genes and target genes (Figure 2). QuantiNova Probe PCR Kit-based real-time PCR analysis was performed for 5 different target genes with each of 5 different reference genes. The results show highly reproducible and comparable results for every possible combination of the genes, with accurate quantification, despite the varying abundances of the analyzed genes.

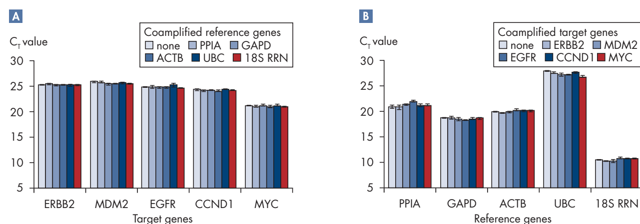

Figure 2. Accurate quantification of 5 different target genes is independent from the chosen reference gene. Real-time PCR analysis of 5 different target genes using the QuantiNova Probe PCR Kit was performed on the Rotor-Gene Q in singleplex reactions, and in duplex reactions in combination with each of 5 different reference genes, using 10 ng cDNA as the template. This resulted in highly reproducible CT-values for all of the **A** target genes and **B** reference genes, despite their varying abundance.

Conclusions

- The QuantiNova Probe PCR Kit gives the robustness, accuracy, and sensitivity required for reliable duplex real-time PCR.
- The kit also provides maximum flexibility in terms of the choice of reference gene for their particular experimental setup.

References

1. Karge, W.H., Schaefer, E.J., and Ordovas, J.M. (1998) Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. Methods Mol. Biol. **110**, 43

2. Thellin, O. et al. (1999) Housekeeping genes as internal standards: use and limits. J. Biotechnol. **75**, 291

3. Dheda, K. et al. (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques **37**, 112

Ordering Information

Product	Contents	Cat. no.
QuantiNova Probe PCR Kit (100)	For 100 x 25 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 1.9 ml RNase-Free Water	208252
QuantiNova Probe PCR Kit (500)	For 500 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 1 ml QN ROX Reference Dye, 1.9 ml RNase-Free Water	208254
QuantiNova Probe PCR Kit (2500)	For 2500 x 25 µl reactions: 15 x 1.7 ml 2x Master Mix, 5 x 500 µl QuantiNova Yellow Template Dilution Buffer, 5 X 1 ml QN ROX Reference Dye, 5 x 1.9 ml RNase-Free Water	208256

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