DNA array and real-time PCR – an optimal combination !
The use of real-time (kinetic) quantitative PCR to validate cDNA array results

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INTRODUCTION

The application of the real-time (kinetic) PCR to amplify cDNA products reversely transcribed from mRNA (RT) and microarray technology are on the way to become routine tools in molecular biology. They are both well suited to study gene expression, but each methodology has its specific advantages and disadvantages. Microarray technology is ideal to screen a lot of genes in one step (>10,000 gene transcripts) and kinetic RT-PCR is very sensitive, highly quantitative and requires up to 1000-fold less RNA. Both allow a relative and accurate quantification of mRNA molecules with a sufficiently high repeatability and low variability.

The simplest detection technique of newly synthesized PCR products in real-time PCR uses SYBR Green I fluorescence dye, that bind specifically to the minor groove dsDNA. The quantification method of choice depends on the target sequence, the expected range of the mRNA amount present in the tissue, the degree of accuracy required, and whether quantification needs to be relative or absolute (*Pfaffl & Hageleit, 2001*). Generally two quantification types in real-time RT-PCR are possible:

- A relative quantification based on the relative expression of a target gene versus a reference gene. This model needs no calibration curve. To investigate the physiological changes in gene expression, the relative expression ratio is adequate for the most purposes.
- An absolute quantification, based either on an internally or an externally calibration curve. Using such a calibration curve, the methodology has to be highly validated and the identical LightCycler PCR amplification efficiencies for standard material and target cDNA must be confirmed (*Pfaffl & Hageleit, 2001*).

A normalisation of the target gene expression with an endogenous standard gene expression is recommended. Therefore mainly non regulated reference genes or housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase (GAPDH), albumin, actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA were applicable. Housekeeping genes are present in all nucleated cell types since they are necessary for basis cell survival. The mRNA synthesis of these genes is considered to be stable and secure in various tissues, even under experimental treatments. But numerous studies have already shown that the mentioned house keeping genes are regulated and vary under experimental conditions. To circumvent the high expenditure of design and production of standard material, as well as optimisation and validation of a calibration curve based quantification model, and finally the needed normalisation of the target transcripts to an endogenous housekeeping gene transcript, an reliable and accurate relative quantification model in kinetic (RT-) PCR was needed.

However accurate quantification of nucleic acids requires a reproducible methodology and an adequate mathematical model for data analysis. The particular topics of the relative quantification in microarray and kinetic PCR technology of a target gene transcript in comparison to a reference gene transcript or housekeeping gene are described herein.

MATHEMATICAL MODELS

A mathematical model for the calculation of relative expression levels (*Pfaffl, 2001*) and the corresponding software REST© is presented (*Pfaffl et al., 2002*).

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

Equation 1: The relative expression ratio **(R)** is calculated from the kinetic PCR efficiencies **(E)** and the crossing point **(CP)** deviation **(ΔCP)** of an unknown sample versus a control. Control levels were included in the model to standardize each reaction run with respect to RNA integrity, sample loading and inter-PCR variations.

E_{target} = real-time PCR efficiency of target gene transcript

E_{ref} = real-time PCR efficiency of reference gene transcript

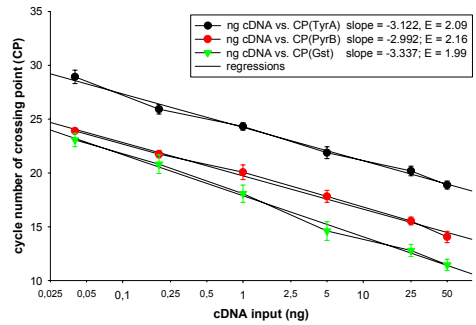
ΔCP_{target} = CP deviation of *control - sample* of target gene transcript

ΔCP_{ref} = CP deviation of *control - sample* of reference gene transcript

real-time PCR amplification efficiencies, variation and linearity

Real-time PCR efficiencies were calculated from the given slopes in LightCycler software. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation *(Rasmussen, 2001)*: $E = 10^{[-1/slope]}$ (Figure 1). In general transcripts show kinetic PCR efficiency rates between E_{min}= **1.7** and E_{max}= **2.1** in the investigated range from 0.40 pg to 50 ng cDNA input with high linearity (Pearson correlation coefficient 0.95>r>0.99). High accuracy and reproducibility (<2.5% variation) were reached in LightCycler® RT-PCR using the established mathematical model.

Figure 1: Determination of real-time PCR efficiencies of reference gene and target genes. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/slope]}$



Relative Expression Software Tool - REST©

Today relative expression is increasingly used, where the expression of a target gene is standardised by a non regulated reference gene. Several mathematical algorithm have been developed to compute an expression ratio, based on real-time PCR efficiency and the crossing point deviation of an unknown sample versus a control *(Livak & Schmittgen, 2001)*. All published equations and available models for the calculation of relative expression ratio allow only for the determination of a single transcription difference between one control and one sample. Therefore a software tool was established, named *REST © (Relative Expression Software Tool),* which compares two groups, with up to 16 data points in sample and 16 in control group, for reference and up to four target genes. The mathematical model used is based on the PCR efficiencies and the mean crossing point (mean CP) deviation between sample and control group. Subsequently the expression ratio results of the four investigated transcripts are tested for significances compared to control on the basis of an *Pair Wise Fixed Reallocation Randomization Test ©*.

$$R = \frac{(E_{target})^{\Delta CP target\ (MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta CP ref\ (MEAN\ control - MEAN\ sample}}$$

Equation 2: The relative expression ratio **(R)** is calculated from the kinetic PCR efficiencies **(E)** and the crossing point **(CP)** deviation **(ΔCP)** of an unknown sample versus a control.

E_{target} = real-time PCR efficiency of target gene transcript

E_{ref} = real-time PCR efficiency of reference gene transcript

ΔCP_{target} = ΔCP of mean $CP_{control}$ – mean CP_{sample} of target gene transcript

ΔCP_{ref} = ΔCP of mean $CP_{control}$ – mean CP_{sample} of reference gene transcript

CONCLUSION

In view of the data provided for linearity and reproducibility, the real-time RT-PCR in combination with the REST© allows for a relative and accurate quantification of low abundant mRNA molecules in various tissues.

Using this approach, to screen the tissue specific expression levels by microarray and confirm the results by kinetic RT-PCR and REST © is a powerful and optimal combination. The advantages of both quantification systems were added - high throughput of the microarray and sensitivity of the real-time RT-PCR. The latest software version of REST and examples for the correct use can be downloaded at: **http://www.wzw.tum.de/gene-quantification/**

References

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