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EDITORIAL

# qRT-PCR: a method and its difficulties

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Today, quantitative reverse transcription polymerase chain reaction (qRT-PCR) is the favored method to quantify gene expression in molecular biology and clinical studies. qRT-PCR analyses can be performed in a high-throughput manner and are very sensitive and specific, cost-effective, and reproducible. Even though this technique is easy to handle, the executer has to pay attention to some critical points. Right from the beginning of proper data generation, it is important to store the samples correctly and to prepare pure and intact RNA. It is, for example, more difficult to obtain good-quality RNA from paraffin-embedded samples than of frozen tissues (Takano et al. 2010). There are RNA protection and preparation kits available that help to maintain the integrity of RNA. Subsequently, an effective reverse transcription reaction (RT) and a specific primer design are indispensable for a high PCR efficiency. For a clear survey, Udvardi's group posted 11 golden rules of performing qRT-PCR analyses and gave a concise support from sample collection to data analysis (Udvardi et al. 2008).

Before performing qRT-PCR analyses, the choice between two detection methods has to be made: nonprobe-based and probe-based assays. Nonprobe-based analyses use an intercalating dye like SYBR® green or ethidium bromide for the target detection. The dye intercalates into all double stranded DNAs formed during the PCR reaction. To exclude the detection of unspecific PCR products, it is necessary to analyze the PCR products further by melting curves or gel electrophoresis. The major advantage of nonprobe-based analyses even in high throughput experiments is the lower investment, because each optimized PCR can easily be converted into a quantitative PCR. In contrast, probe-based detection needs an additional oligonucleotide that binds internal to the amplified target sequence and contains a quencher and reporter. The probe has to be designed for every target sequence, and this, in turn, guarantees a higher specificity for the detection system. Several different probe structures can be used for this method: TaqMan®, molecular beacons, or Scorpion Primers.

A crucial point of qRT-PCR analyses is the normalization of the results that, improperly done, can have a profound influence on study conclusions (Ferguson et al. 2010). Even though normalization is a highly discussed topic, mostly neither editors nor reviewer demands on the proof of a suitable data evaluation. Current used normalization strategies range from standardization of sample size, like tissue weight or RNA amount, to internal and external standards. When normalizing with external standards, the absolute copy number of the target transcripts can be determined. The standard curve can either be generated from diluted plasmid DNA (Li and Wang 2000) or from in vitro-transcribed RNAs (Workenhe et al. 2008). The quantification is based on the assumption that plasmid DNA and cDNA [reverse transcribed from messenger RNA (mRNA)] have the same amplifications rates. In contrast to the external control, it is also possible to use coamplified internal controls. This normalization strategy to one or more internal controls is commonly used (Vandesompele et al. 2002). The principle of this method is based on the quantification of the mRNA of an internal reference gene that undergoes the same procedures and conditions as the mRNA of interest. Using this kind of normalization, it is absolutely essential to search for a reference gene whose expression is

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neither influenced by the experimental conditions (Weiss et al. 2012) nor differently regulated in various organs, tissues, or cell lines that might be of interest (Cabiati et al. 2012). So-called housekeeping genes (HKGs) are often used as internal controls for qRT-PCR analyses. HKGs are defined to be constitutively expressed in many tissues, organs, and organisms and are essential for cell survival. However, it is already known that commonly used reference genes, like glyceraldehyde-3-phosphate dehydrogenase, beta-2-microglobulin, or rRNA, can be regulated in various tissues or under certain experimental conditions (Guenin et al. 2009; Stahlberg et al. 2004). In this issue, Weiss et al. (2012) show a concentration-dependent regulation of several HKGs by rifampicin. They demonstrate that the regulation of those HKGs, used for normalization, leads to misinterpretation of qRT-PCR-data. A first advice, whether a reference gene is regulated by different conditions, in organs, or cell types, may be the $C_t$ values measured. The $C_t$ values should not differ for more than 0.5 numerical values when conditions like RNA quality and amount, RT efficiency, and the quantity of the performed experiments are considered equally. If reference genes are obviously regulated, microarray analyses can be performed to get an idea of unregulated genes in the experimental settings (Coito et al. 2012). However, it is necessary to confirm the microarray results again by qRT-PCR to verify the $C_t$ values. First indications can be given by programs like RefGenes from Genevestigator that compares already demanded microarray data from experiments with similar conditions with the own data (http://www.refgenes.org).

To simplify the search for reference genes programs like GeNorm, BestKeeper or Normfinder have been designed. Those will calculate adequate reference genes, after uploading the data of several experimental conditions. The idea of the often used GeNorm is the pairwise comparison approach of unregulated genes (Vandesompele et al. 2002). It defines the internal control gene-stability measure for every HKG variation and eliminates the worst scoring HKG in each step. The program lists a ranking of the stability of each HKG. A disadvantage of this knockout method is the elimination of a strong HKG, when compared to another strong HKG. This leads to a possible loss of stable HKGs in early steps. The program BestKeeper is based on a similar analysis, but additionally determines further stably expressed genes. So if BestKeeper determines unregulated target genes, they can be used as an expression standard too (Pfaffl et al. 2004). Normfinder, in contrast, is a model-based approach. It calculates the stability value for all candidate genes and can calculate it for several subgroups like tumor vs. normal tissue. Therefore, the program puts the genes into context and ranks the HKGs due to their “minimal estimated intra- and intergroup variation” (Andersen et al. 2004).

In conclusion, qRT-PCR as a sensitive tool for the determination of mRNA expression requires critical screening of normalization for the experimental outcome. The tissue storage and preparation should be standardized and optimized for each sample. The use of well-established reference genes needs to be tested for every new experimental condition. Several documents, like the MIQE guidelines (Bustin et al. 2009), and computer programs can additionally help to choose the reference genes with the highest stability in the experiment (http://www.gene-quantification.de/). Due to varying reference gene expression, the determination of multiple internal controls or a triplex detection of the RNA of interest and internal and external controls (Vandenbussche et al. 2010) is recommended.

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