

# Chapter 2

## The Use of Real-Time Quantitative PCR for the Analysis of Cytokine mRNA Levels

Maria Forlenza, Thomas Kaiser, Huub F.J. Savelkoul,  
and Geert F. Wiegertjes

### Abstract

Over the last decade, real-time-quantitative PCR (RT-qPCR) analysis has become the method of choice not only for quantitative and accurate measurement of mRNA expression levels, but also for sensitive detection of rare or mutated DNA species in diagnostic research. RT-qPCR is based on the standard principles of PCR amplification in addition to the use of specific probes or intercalating fluorescence dyes. At the end of every cycle, the intercalating dye binds to all double-stranded DNA. There is a quantitative relationship between the amount of starting DNA and the amount of amplification product during the exponential phase. However, to obtain meaningful RT-qPCR data, the quality of the starting material (RNA, DNA) and the analysis method of choice are of crucial importance. In this chapter, we focus on the details of RNA isolation and cDNA synthesis methods, on the application of RT-qPCR for measurements of cytokine mRNA levels using Sybr-Green I as detection chemistry, and finally, we discuss the pros and cons of the absolute quantification versus relative quantification analysis. RT-qPCR is a powerful tool, but it should be “handled” with care.

**Key words:** Real-time-quantitative PCR, Absolute quantification, Relative quantification, Primer efficiency, Housekeeping gene

---

### 1. Introduction

Over the last decade, real-time-quantitative PCR (RT-qPCR) analysis has become the method of choice not only for quantitative and accurate measurement of mRNA expression levels, but also for sensitive detection of rare or mutated DNA species in diagnostic research (1, 2). RT-qPCR is based on the standard principles of PCR amplification in addition to the use of specific probes or intercalating dyes. Various probe systems are available among which

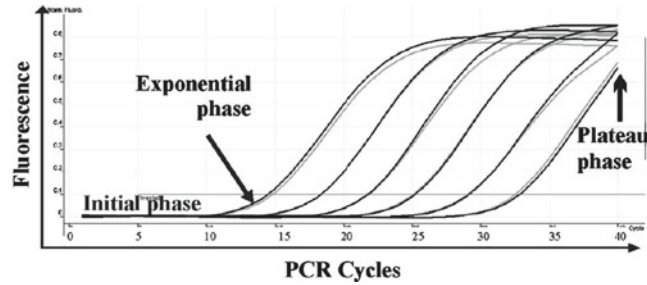


Fig. 1. A typical RT-qPCR profile can be divided in the initial, exponential, and plateau phases.

TaqMan probes, Molecular Beacons, MGB probes, and others increasing specificity and sensitivity of the real-time assays. RT-qPCR, using intercalating dyes that become fluorescent upon binding to double-stranded (ds) DNA, has the advantage of running melting curve analysis after each run in order to check specificity. In most cases, Sybr-Green I is used, but other dyes are available, including Eva-Green, Syto9, etc.

Under optimal conditions, every PCR cycle should result in a doubling of the amplification product. At the end of every cycle, the intercalating dye binds to all double-stranded DNA. Ideally, the increase in amount of template is directly proportional to the increase in fluorescence. Fluorescence data are collected during each cycle allowing for real-time monitoring of amplification. A typical RT-qPCR profile is shown in Fig. 1: it can be divided in the initial, exponential, and plateau phases. The exponential phase of the amplification provides the most useful and reproducible data. *There is a quantitative relationship between the amount of starting DNA and the amount of amplification product during the exponential phase.*

The number of cycles required for a sample to rise above the background fluorescence and reach the threshold level is called Ct-value (threshold cycle). The threshold is set at a level, where the rate of amplification is greatest during the exponential phase, allowing for the most accurate and reproducible results. An advantage of RT-qPCR over conventional PCR is the possibility to assess the amplification efficiency ( $E$ ). Particularly, when the expression profile of more genes needs to be compared, it is important to take the efficiency into account and adjust for differences between different genes to be compared. In addition, at the end of every run, a *melting curve analysis* can be performed to assess amplification specificity. Taken together, this leads to increased sensitivity, specificity, and efficiency of the PCR analysis. To obtain meaningful RT-qPCR data, the quality of the starting material (RNA, DNA) and the analysis method of choice are of crucial importance. In this chapter,

we focus on the details of *RNA isolation* and *cDNA synthesis* methods, the application of RT-qPCR for measurements of *cytokine mRNA levels* using *Sybr-Green I* as detection chemistry, and finally, we discuss the pro and contra of the *absolute quantification* versus *relative quantification* analysis.

### 1.1. Absolute Quantification

Absolute quantification analysis ideally determines the absolute copy number of a gene of interest (GOI) in an unknown sample. The unknown sample is compared to a standard curve with known concentrations of template. In most cases, recombinant plasmid DNA (recDNA), cDNA, recRNA, pooled samples, or PCR products are used for this purpose. Therefore, *the accuracy of the absolute quantification assay entirely depends on the accuracy of the standard* (3). No matter how accurate the concentration of the standard material is *the final result is always expressed relatively to a defined unit of interest*: e.g. copies per ng of total RNA, copies per cell, copies per gram of tissue, copies per mL blood. When absolute changes in copy numbers are important, the denominator has to be shown to be *absolutely stable* across the comparison. Although the word “absolute” suggests an exact measurement, one has to be aware that absolute quantification is relative to the standards used.

### 1.2. Relative Quantification

Relative quantification analysis determines the levels of expression of a GOI and expresses it relative to the levels of an internal control or reference gene (RG). Results are given as ratio of GOI versus one or more RGs (4). In this type of analysis, the function of the RG is to *normalize* the data for differences in RNA (DNA) quantification and template input. Therefore, expression of the RG has to be analyzed in the same sample as the GOI and can be co-amplified in the same tube as a multiplex assay (probes) or the same sample should be used in separate tubes as a simplex assay (Sybr-Green I).

Reference genes are genes that are not affected by the treatment in any way and are constant under the tested conditions. Hence, *the reliability of the relative quantification analysis is strongly dependent on the stability of the RG*. Several tools are available for the determination of the best RG: *TATA Biocenter AB*: <http://www.tataa.com/Products/Human-Endogenous-Control-Panel.html>; *geNorm* (5): <http://medgen.ugent.be/~jvdesomp/genorm/>; *BestKeeper* (6): <http://www.gene-quantification.info>. We have extensive experience with the BestKeeper software.

### 1.3. Real-Time PCR Cyclers

Most RT-qPCR cyclers make use of a solid block (96 or 384 wells) for thermal cycling while others use hot and cooled air. Most of the solid block-based real-time instruments are affected by thermal variation across the block and by differences in illumination and

optical signals detected from each sample. Both aspects greatly contribute to well-to-well variability. Two air-based cyclers employ a rotary design using capillaries or plastic tubes and one of them uses a centrifuge, which guarantees optimal thermal and optical uniformity. Samples are continuously rotating in the thermal chamber, guaranteeing minimal temperature variation between tubes in contrast to positional effects, such as the recognized “edge effect” observed in block-based designs. In addition, every tube moves past the identical excitation light source and detection pathway, which guarantees optical uniformity. In our laboratory, we have extensive experience with the Rotor-Gene 6000™.

---

## 2. Materials

### **2.1. RNA Isolation and cDNA Synthesis**

1. RNA isolation, including on-column DNase treatment: RNeasy Mini Kit and RNase-free DNase set (QIAGEN).
2. cDNA synthesis, including DNase treatment: DNase I, Amplification Grade; Superscript™ III First Strand Synthesis Systems for RT-PCR Systems (Invitrogen).
3. Nuclease-free water (Promega).
4. RT-qPCR Master mix: ABsolute™ QPCR SYBR® Green Mix (ABgene).
5. NanoDrop spectrophotometer (Thermo Scientific).
6. Thermal cycler: Rotor-Gene 6000™ (Corbett Research).

More detailed information to any RT-qPCR topic can be found on the following Web site: <http://www.gene-quantification.info>

### **2.2. Plasmid Construction and Isolation**

1. Luria Bertani (LB) medium (1 L).
2. LB plates.
3. *E. coli* JM109 High Efficiency Competent Cells (Promega).
4. pGEM-T easy Ligation Kit (Promega).
5. QIA prep Spin Miniprep kit (QIAGEN).
6. Gel Extraction Kit (QIAGEN).
7. Ampicillin.
8. X-gal.
9. IPTG.
10. SOC medium.
11. 100% glycerol.
12. Agarose.

### 3. Methods

#### 3.1. RNA Isolation and Quantification

Isolation and quantification of good-quality RNA (see Note 1) are of extreme importance to obtain meaningful gene expression data by RT-qPCR. Several commercial kits are available; for RNA isolation, from small (30 mg), fresh-frozen or RNA-*later* stored tissue samples and from primary cells or cell lines ( $10^7$  cells), we obtained high-quality results with the RNeasy mini kit from Qiagen.

1. Isolate RNA according to the manufacturer's instructions. Work fast, clean, wear gloves, and use RNase-free tubes and tips. To reduce genomic DNA (gDNA) contaminations, include an on-column DNase digestion step. Elute RNA in 30–50  $\mu$ L RNase-free water.
2. Use 1–2  $\mu$ L of the eluted sample to determine RNA concentration (OD measurement at 260 nm) and RNA quality (OD 260/280 ratio) with the NanoDrop spectrophotometer. An OD 260/280 ratio greater than 1.8 is usually considered an acceptable indicator of good RNA quality. The presence of gDNA in the sample leads to an overestimation of the RNA concentration.
3. RNA integrity and the absence of gDNA can be assessed by loading 1–2  $\mu$ L of RNA sample on a 1% agarose gel. Two major bands corresponding to the 28S and 18S rRNA should be clearly visible. In case of gDNA contaminations, an additional band of higher molecular weight than the two rRNA bands can be observed.

#### 3.2. cDNA Synthesis

Several kits are available for cDNA synthesis. We routinely use the SuperScript<sup>™</sup> III First strand cDNA synthesis kit with random primers from Invitrogen.

1. Prior to cDNA synthesis from 1  $\mu$ g of total RNA (see Note 2), perform a second DNase digestion step using the DNase I Amplification Grade Kit (Invitrogen).
2. Proceed with the cDNA synthesis protocol according to the manufacturer's instructions. For each sample, always include a control for gDNA contaminations: in this sample, the same amount of RNA is used but no reverse transcriptase is added to the mix (–RT control).
3. After cDNA synthesis, the final volume for each sample is 20  $\mu$ L. We routinely bring the volume up to 100  $\mu$ L and consider this our stock sample solution. Depending on the organ or cell type, we further dilute the stock five to ten times. This allows performing up to 200 reactions for each sample when using 5  $\mu$ L of template in each PCR reaction.

### **3.3. Construction of Recombinant Plasmid DNA**

The calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules, e.g. recDNA, gDNA, RT-PCR product, commercially synthesized, big oligonucleotide (see Note 3). In this section, we describe how to construct a recombinant plasmid DNA containing the sequence of any GOI.

1. Design primers to amplify a large (500–1,000 bp) fragment of the gene. The region should of course contain the sequence to which the primers designed for RT-qPCR anneal. Amplify the large product by conventional PCR or reverse transcriptase-PCR.
2. Gel purify the product using the QIAgen Gel Extraction Kit and elute in 30  $\mu$ L of water.
3. Ligate the product into the vector by combining 3.5  $\mu$ L of the gel-purified product to 5  $\mu$ L of 2 $\times$  ligation buffer, 0.5  $\mu$ L (25 ng) of pGEM-T easy, and 1  $\mu$ L (3 UI) of T4 DNA ligase (all reagents in the easy ligation kit). Mix by pipetting, and incubate for 1 h at room temperature or overnight at 4°C for the maximum number of transformant.

### **3.4. Amplification and Quantification of recDNA**

1. Prepare LB agar plates containing ampicillin, X-Gal, and IPTG.
2. Centrifuge the ligation reactions briefly. Add 2–5  $\mu$ L of each ligation reaction to a sterile 10-mL tube on ice.
3. Thaw one vial (200  $\mu$ L) of JM109 High Efficiency Competent Cells on ice. When just thawed, mix the cells by gently flicking the tube. Carefully transfer 50  $\mu$ L of cells to the ligation tube from step 2. Gently flick the tube and incubate on ice for 20 min.
4. Heat shock the cells for 45–50 s in water bath at exactly 42°C. DO NOT SHAKE. Immediately return the tube to ice for 2 min.
5. Add 950  $\mu$ L room temperature SOC medium to each reaction tube. Incubate for 1.5 h at 37°C with shaking (~150 rpm).
6. Transfer the total volume of the transformation reaction to an Eppendorf tube, and centrifuge for 10 min at 350*g*. Remove 900  $\mu$ L of medium and resuspend the bacterial pellet in the remaining 100  $\mu$ L. Spread 90 and 10  $\mu$ L of cell suspension onto two LB agar plates containing ampicillin, X-Gal, and IPTG and incubate overnight at 37°C.
7. With a sterile pipette tip, tick pick 5–8 white colonies and transfer each of them in 4 mL LB medium containing ampicillin (50  $\mu$ g/mL). Grow overnight with shaking at 300 rpm.
8. Isolate plasmid from 3 mL of the overnight culture using the QIAgen QIA prep Spin Miniprep kit. Elute plasmid in 50  $\mu$ L of water.

9. Make glycerol stocks by combining the remaining 1-mL overnight culture to 200  $\mu$ L 100% glycerol.
10. Load 1–2  $\mu$ L of isolated plasmid on a 1% agarose gel. Three bands of high molecular weight corresponding to the linear, circular, and supercoiled forms of the plasmid should be visible.
11. Linearize the plasmid by combining 30  $\mu$ L of purified plasmid to 3  $\mu$ L of restriction enzyme of choice, 5  $\mu$ L of the appropriate 10 $\times$  reaction buffer, and water up to a final volume of 50  $\mu$ L.
12. Gel purify the linearized plasmid using the QIAgen Gel Extraction Kit and elute in 30  $\mu$ L of water.
13. Determine plasmid concentration using the NanoDrop. Take an average out of at least five measurements (better ten) and perform the measurement at multiple template dilutions. *The concentration of the plasmid has to be calculated very accurately because this measurement determines the outcome of the absolute quantification analysis.* For use in RT-qPCR, prepare the plasmid as described below.

### 3.5. Calculation of Plasmid Copy Number and Preparation of the Standard Curve

Once the size of the plasmid containing the GOI is known, it is possible to calculate the number of grams/molecule, also known as copy number, as in the following example:

Weight in Daltons (g/mol) = (bp size of plasmid + insert)(330 Da  $\times$  2 nucleotides/bp).

Ex. g/mol = (5,950 bp)(330 Da  $\times$  2 nucleotides/bp) = 3,927,000 g/mol.

Hence, (g/mol)/Avogadro's number  $6.02214199 \times 10^{23}$  = g/molecule = copy number.

Ex.  $3,927,000 \text{ (g/mol)} / 6.02214199 \times 10^{23} = 6.52 \times 10^{-18} \text{ g/molecule}$ .

The precise number of molecules can be determined as follows:

Concentration of plasmid (g/ $\mu$ L)/copy number.

Ex.  $(3 \times 10^{-7} \text{ g}/\mu\text{L}) / (6.52 \times 10^{-18} \text{ g/molecule}) = 4.6 \times 10^{10} \text{ molecules}/\mu\text{L}$ .

Once the number of molecules in 1  $\mu$ L of linearized plasmid solution is calculated, prepare standard dilutions to obtain an X plasmid copy number in 5  $\mu$ L of water. Accurate pipetting is essential because the standards must be diluted over several orders of magnitude. It is recommended to divide standards into small aliquots, store at  $-80^\circ\text{C}$ , and thaw only once before use.

### 3.6. Primer Design

The design of specific primers that work at a good efficiency is of crucial importance in RT-qPCR. Use the Primer3 program ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) to design primers of a length of 18–22 bp, with an annealing temperature of  $60^\circ\text{C}$  and a minimum self and 3' complementarity. To accomplish rapid quantification, short PCR cycling (45–75 s),



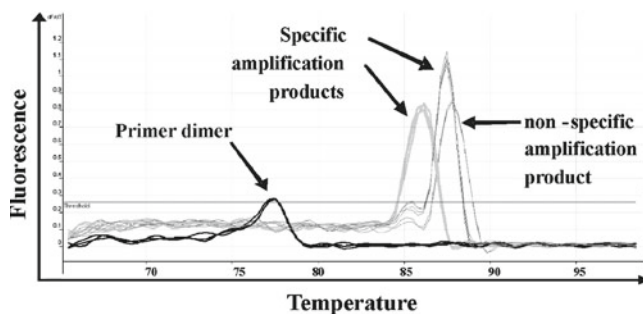


Fig. 2. Melting curve profile of PCR products amplified with three different primer sets. *Light grey*: Four PCR products each showing the same specific melting peak. *Dark grey*: Four PCR products of which three showing a specific melting peak and a fourth one being a non-specific amplification product with a different melting temperature. *In black*: Amplification with the third set of primers resulted only in primer dimer formation.

and efficient PCR conditions, the optimal length of the PCR product is 100–200 bp. Use the OligoAnalyzer program (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx?c=EU>) to verify that the primers have low self- and hetero-complementarity. To increase the annealing temperature of primers, to improve the specificity of allele-specific primers, or for single-nucleotide polymorphism (SNP) analysis, the incorporation of locked nucleic acid (LNA) modifications can be of great advantage (7, 8). Software program to estimate melt behaviours of a template is POLAND MELTSIM (<http://www.bioinformatics.org/meltsim/wiki/>).

### 3.7. PCR Profile and Melting Curve Analysis

A typical PCR profile includes an initial denaturation step of 10–15 min at 95°C, depending on the Taq-Polymerase (see Note 4), followed by 35–40 cycles, including 95°C for 5–15 s (denaturation), 60°C for 15–30 s (annealing), and 72°C for 15–30 s (elongation). This profile is a general suggestion and the annealing temperature has to be verified. At the end of the run, a melting step needs to be performed to assess amplification specificity (see Note 5, Fig. 2). Each PCR product has a specific melting temperature, resulting in a single melting peak with no additional peaks at lower melting temperatures. Additional peaks can be primer dimers or unspecific products due to excessive amount of primers in the reaction, low annealing temperature, too high MgCl<sub>2</sub> concentration, or too long hold times. Primer dimers' formation can be reduced or eliminated by accurate design of the primers and optimization of primer concentration. When using a primer set for the first time, despite the presence of only one amplification peak, it is advised to sequence at least once the amplification product to confirm sequence specificity.

### 3.8. Optimization of Primer Concentration

Select a cDNA template or recDNA containing the sequence of the GOI. Prepare a master mix containing 7 µL of 2× Sybr-Green I Mix and 5 µL of DNA. Aliquot 12 µL of the master mix into reaction



tubes and add 1  $\mu\text{L}$  of each primer to give final concentrations as outlined in the table below. The final reaction volume is 14  $\mu\text{L}$  (see Note 6). The primer stock concentrations are 1.4, 4.2, and 7  $\mu\text{M}$  and give final concentrations of 100, 300, and 500 nM, respectively.

Forward primer (nM)			
Reverse primer (nM)	100	300	500
100	100/100	300/100	500/100
300	100/300	300/300	500/300
500	100/500	300/500	500/500

We usually find 300 nM the optimal concentration for both forward and reverse primers. As a general guideline, choose the primer combination which gives the lowest Ct value for the same amount of template and does not lead to primer dimer formation. In every run, *always include a non-template control (NTC)*, where the template is replaced by the same amount of water, in order to test for primer specificity and contaminations.

**3.9. Determination of Primer Amplification Efficiency**

Depending on the subsequent method of analysis, there are several ways to determine primer amplification efficiencies. The most commonly used is the standard curve method: a dilution series of a reference template or pooled samples of unknown concentration is generated. The reference sample can be cDNA or recDNA (of unknown concentration) that contains the target gene. The units used to describe the concentration of the dilution series are relative, as long as they reflect the dilution factor of the standard curve (Fig. 3).

Set the threshold just above the take-off point of the reactions (if the result for more genes over different experiments need to be compared, set the threshold at the same level for all genes, for example 0.1). Record the Ct values and plot them against the log template concentration. Use the slope of the regression line to calculate the amplification efficiency for each primer according to the following formula:  $E = 10^{(-1/\text{slope})}$ . The optimal amplification efficiency of a reaction is 2, but we consider *E* values between 1.7 and 2 as acceptable, as long as the reproducibility over several runs as well as the replicates is good. Usually, all RT-qPCR software provide this type of calculations (see example in Table 1 and Fig. 4). In general, it is important that *the amplification efficiency of the reference template reflects the amplification efficiency of the unknown sample*.

**3.10. Relative Quantification Analysis**

Relative quantification is the method of choice for RT-qPCR analysis when investigating physiological changes in gene expression levels. It does not require standard curves with known concentration of templates and results are given as the ratio (*R*) of GOI versus

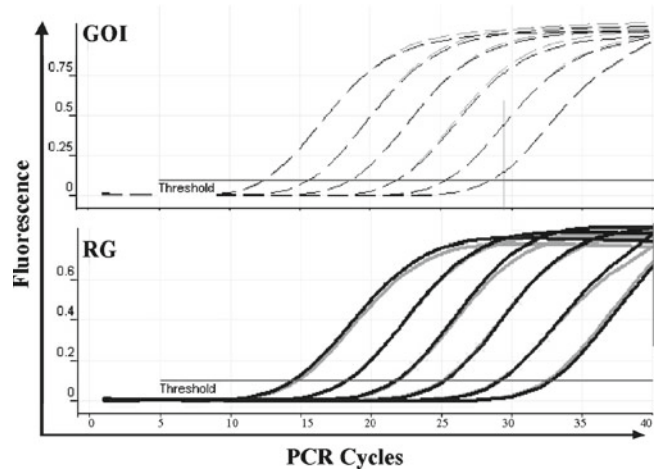
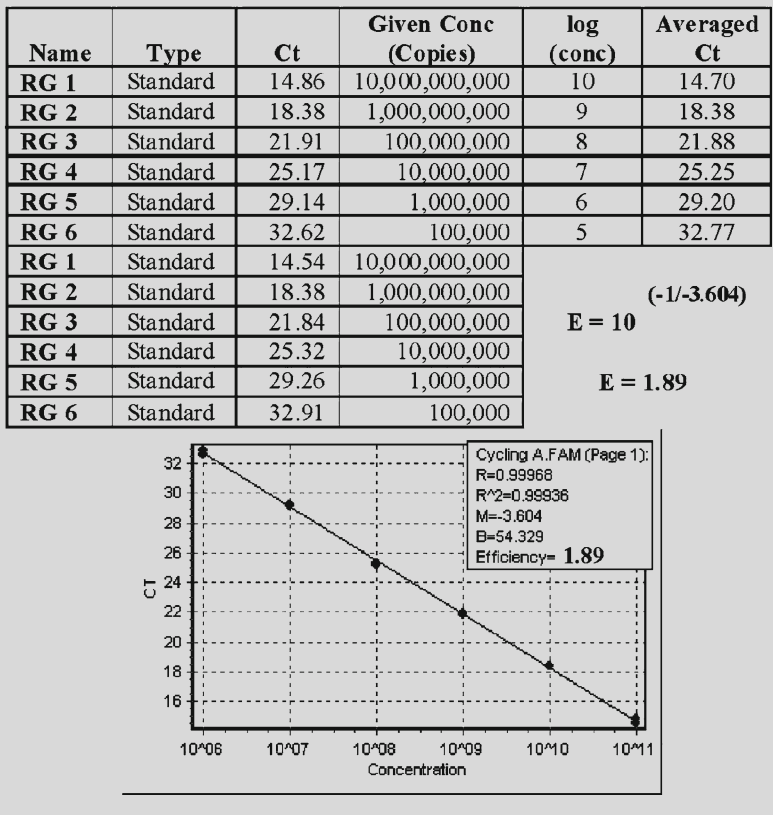


Fig. 3. Standard curve of a tenfold dilution series of a reference cDNA sample used to calculate the amplification efficiency of the primer sets for the RG and GOI.

**Table 1**  
Results obtained from the RG standard curve described in Fig. 3. By plotting the averaged Ct values from duplicate samples against the log of the given concentration, the corresponding standard curve will be obtained as shown in this table.



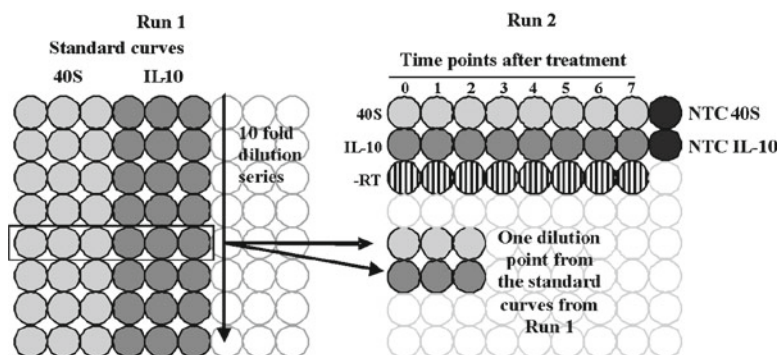


Fig. 4. Possible RT-qPCR set up for relative quantification of IL-10 mRNA expression levels. A first run, where a standard curve for each of the analyzed genes is amplified has to be performed when analyzing the data using  $\Delta\Delta C_t$  and *Pfaffl* method (see text). Particularly, for the validation experiment required for the  $\Delta\Delta C_t$  method, it is important the template to be the same for each gene which needs to be compared. In a second run (the experimental run), amplify the RG (40S) and the GOI (IL-10) in each of the samples under investigation. Always include a non-template control (NTC), where water substitutes the template, and a control for genomic contamination (-RT). When a standard curve should be imported from a previous run, include a triplicate sample of one dilution point of the same standard curve in the current run (see Note 7).

one or more RGs. To date, several mathematical models have been developed and can be generally divided into two major categories: *without* and *with* primer efficiency correction. In this section, we provide examples on how to analyze an experiment applying both of those methods. For example, we want to determine the fold change in interleukin-10 (IL-10) mRNA expression at various time points after treatment. The 40S ribosomal protein S11 is used as RG. Set up the first run of the day by amplifying a standard curve for each target gene using a template cDNA or recDNA of unknown concentration (Fig. 4; it might not be necessary to run a standard curve every time depending on the chosen method of analysis). Set up a second run, where in separate tubes the 40S and IL-10 genes are amplified for each of the samples under investigation. Include a triplicate sample of one dilution point of the same standard from the first run. Analyze the results according to one of the methods outlined below.

### 3.10.1. Relative Quantification Without Efficiency Correction: $\Delta\Delta C_t$ Method

The  $\Delta\Delta C_t$  method (9) is based on the assumption that the primers of the GOI will have the same amplification efficiency as the primers for the RG. This assumption needs to be validated at least once before proceeding with the analysis of the experiment. See Note 8 for instructions on the *validation experiment*. In case of positive results from the validation experiment, proceed as follows.

- Set the threshold to 0.1 for all genes.
- Export the  $C_t$  values to Excel.

- Select the sample at time point 0 (zero) as the *calibrator* (the calibrator is usually an untreated, unhandled sample).
- Apply the following formula:

$$\Delta\Delta Ct = (Ct_{IL-10} - Ct_{40S})_{(sample)} - (Ct_{IL-10} - Ct_{40S})_{(calibrator)},$$

$$R_{(IL-10)} = 2^{-\Delta\Delta Ct}.$$

This method has the advantage that standard curves are required only once for the validation experiment and allows for normalization relative to an internal reference gene. However, the assumption that different primer sets will perform with the same amplification efficiency over different runs and over different templates might not always be valid. Therefore, the efficiency of all RG and GOI should be checked regularly, as changes in reagents, concentrations, calibrator, etc. could influence the efficiency of one or various genes differently.

### 3.10.2. Relative Quantification with Efficiency Correction: The Pfaffl Method

This method does not require the amplification efficiency of different primer sets to be similar; it rather takes into account the possibility that the efficiencies can be different and offers a way to correct for such differences (4, 10). Optimally, a standard curve for each of the target genes is amplified in the same run together with the unknown samples. However, when a large number of samples and numerous genes need to be analyzed, standard curves for several genes can be amplified in the first run of the day or even on a different day (Fig. 4).

- Set the same threshold for all genes to be analyzed (i.e. 0.1) and record the amplification efficiency for each primer set as described in the previous paragraph.
- In the experimental run, it is possible to either import the standard curve from the previous run and ask the software to adjust it to the standard in the current run (Run 2 in Fig. 4) or the threshold can be directly set manually to 0.1.
- Export the Ct values to Excel.
- Select the sample at time point 0 (zero) as calibrator and apply the following formula:

$$R_{IL-10} = \frac{E_{(IL-10)}^{(Ct_{IL-10}(calibrator) - Ct_{IL-10}(sample1-7))}}{E_{E(40S)}^{(Ct_{40S}(calibrator) - Ct_{40S}(sample1-7))}}.$$

The Pfaffl method is a modification of the  $\Delta\Delta Ct$  method with the obvious advantage that it does take into account differences in amplification efficiencies between primer sets.

In order to obtain direct and valuable statistical information, it is possible to import the above-mentioned data in the gene

quantification software called Relative Expression Software Tool (REST, freely available at <http://rest.gene-quantification.info>). This software uses the Pfaffl formula and generates statistical data, including the standard error and the confidence interval, by using randomisation tests via hypothesis testing  $P(H1) = \text{difference between sample and control is due only to chance}$ .

**3.10.3. Relative Quantification with Efficiency Correction: “Sigmoidal” or “Logistic” Curve Fitting Models**

To date, several methods have been developed to calculate the amplification efficiency of each primer set in each single sample (11–13). The great advantage of all these methods is that they do not require the preparation of standard curves or validation experiments and no assumption has to be made regarding the amplification efficiency of each primer set over different runs, templates, or master mixes. The method developed by Corbett Research has been incorporated in the Rotor-Gene 6000 software under the “Comparative Quantitation” analysis option and we routinely use it for our relative quantification of gene expression. We directly apply the set up of Run 2 in Fig. 4 (without the need of the standard samples).

- The Ct values and the amplification efficiency for each sample are directly obtained from the software and exported to Excel.
- The average amplification efficiency ( $E_A$ ) for each primer in each run is calculated and the relative fold change for each GOI is calculated according to the Pfaffl formula as above.
- It often happens that the analysis of one large experiment cannot be completed within one run. In that case, we calculate the  $E_A$  of each primer set over the whole experiment (two, three, or more runs). To reduce variation between runs, we usually prepare one master mix for each primer set which is enough for all runs of the day and not one master mix for each run. By doing so, we observe only a  $\pm 0.02$  variation in  $E_A$  for each primer set between two, three, or more runs on a single day.

Before using a new primer set for the first time, we perform a dilution series of a cDNA sample containing the target gene. This provides us with an estimation of the amplification efficiency and the melting curve analysis provides us the specificity of the assay.

**3.11. Absolute Quantification Analysis: External Standard Curve Model**

Absolute quantification refers to an analysis, where unknown samples are compared to a standard curve of cDNA, recDNA, or recRNA, where the absolute concentration is known. Especially for absolute quantification analysis, the standard curve for the target gene should be amplified in the same run together with the unknown samples. However, when a large number of samples and numerous genes need to be analyzed, it is possible to import a standard curve from a previous run.

- Standard curves for several GOI can be amplified in the first run of the day and in every subsequent run, together with the unknown samples. A triplicate of one dilution point of the standard curve should be included.
- At the end of the run, ask the software to import the standard curve for the GOI from a previous run and adjust it to the standard in the current run (see Note 7). Read the absolute copy number given by the software.
- Alternatively, it is possible to export data to excel and perform the quantification analysis by plotting the Ct values of the unknown sample against the standard line obtained by plotting the Ct values and the log concentration of the recDNA as described before.
- Express data as GOI (copy number)/ $\times$ ng total RNA.
- To normalize data and correct for variations in template input, a normalizer (RG) is used. In this way, the absolute copy number of an RG and GOI in an unknown sample is determined from the standard curve. The absolute value obtained for the GOI is divided by the absolute value obtained for the RG in the same sample. Obtained are the normalized data of the GOI in the unknown sample. *The quality of the gene quantification data cannot be better than the quality of the denominator. Any variation in the denominator obscures real changes, produces artificial changes, and wrongs quantification results.*

When optimized, standard curves are highly reproducible and allow the generation of highly specific, sensitive, and reproducible data. However, the external standard curve model has to be thoroughly validated as the accuracy of absolute quantification in real-time reverse transcriptase-PCR depends entirely on the accuracy of the standards. Standard design, production, determination of the exact standard concentration, and stability over long storage time are not straightforward and can be problematic.

### **3.12. Technical or Biological Replicates?**

Depending on the applications, the use of technical and biological replicates or both has to be considered. A technical replicate refers to a sample, for example a piece of tissue, from which the RNA isolation and cDNA synthesis has been performed more than one time under the same identical conditions. This type of replicate tells us something about the variation in the chemistry we are using. Often, the same cDNA sample is analyzed in triplicate in one RT-qPCR run. This type of technical replicate only tells something about the pipetting skills of the operator and the accuracy of the PCR instrument (see also Subheading 1.3), but should absolutely NOT be considered for statistical analysis. Biological replicates refer to the application of the same treatment to two or more samples. From each of the samples, the RNA isolation and cDNA

synthesis are performed independently but under identical conditions. Each of the obtained cDNA samples can be analyzed once by RT-qPCR.

Both types of replicates (technical or biological) provide information about the experimental variation and allow statistics to be applied to identify differences in expression levels between samples. Being a beginner, it is a good practice to include technical replicates to test for pipetting skills. When testing the amplification efficiency of a new primer set, it is advisable to include at least a triplicate of each dilution point. When investigating the effects of a treatment, the use of biological replicates we think is of greater value (14).

For example, in an in vitro experiment, cells are incubated in the presence or absence of a stimulus. The treatment is repeated in at least three replicate wells. Each of the three wells is a biological replicate; however, the cells are derived from a single individual. More relevant would be to repeat the same in vitro experiment on cells isolated from three different individuals, each of them being a biological replicate.

---

## 4. Notes

1. The extraction and purification procedure of total RNA must fulfill the following criteria: free of protein (absorbance 260/280 nm); free of genomic DNA; should be non-degraded (28S:18S ratio should be roughly between 1.8 and 2.0, with low amount of short fragments); free of enzymatic inhibitors for RT and PCR reaction, which is strongly dependent on the purification and clean-up methods; free of any substances which complex essential reaction cofactors, like  $Mg^{2+}$  or  $Mn^{2+}$ ; free of nucleases for extended storage (15).
2. From 0.1 ng up to 5  $\mu$ g, total RNA can be transcribed into cDNA using this kit. Optimally, 1  $\mu$ g of total RNA is used. In general, it is important to use the same amount of starting RNA material for each sample within the same experiment. This greatly reduces the sample-to-sample variation due to differences in cDNA synthesis efficiency and simplifies the subsequent analysis, particularly when absolute quantification is used. In some cases, not all samples (within the same experiment) would yield RNA amounts sufficient to use 1  $\mu$ g of RNA/sample; it is possible to lower the amounts down to 0.1  $\mu$ g, but again this amount should be used for all samples within the same experiment.
3. Cloned recDNA and gDNA are very stable and generate highly reproducible standard curves even after a long storage time.



Furthermore, the longer templates derived from recDNA and gDNA mimic the average native mRNA length of about 2 kb better than shorter templates derived from RT-PCR product or oligonucleotides. A problem with DNA-based calibration curves is that they are subject to the PCR step only, unlike the unknown mRNA samples that must first be reverse transcribed. This increases the potential for variability of the RT-PCR results and the amplification results may not be strictly comparable with the results from the unknown samples (3).

4. The initial denaturation time depends on the type of *Taq*-Polymerase present in the master mix. We strongly advise Hot-Start *Taq*-Polymerases that require 2 to 15 min at 95°C, depending on the *Taq*-Polymerase. This allows performing the preparation and aliquoting of the master mix on the bench at room temperature.
5. At the end of a run, after the last annealing step, all amplification products are present as double-stranded DNA and Sybr Green I is bound to it. During the melting step, the decrease in fluorescence is measured due to melting of dsDNA products and consequent release of the fluorescent dye. Each product melts at a specific temperature. Primer dimers usually have a lower melting temperature than PCR products ranging between 80 and 200 bp.
6. Usually, companies advise a final volume of 50  $\mu\text{L}$ , but the reaction can easily be scaled down to save costs. We always try to add at least 5  $\mu\text{L}$  of template. Lower volumes might increase the chance of pipetting errors.
7. The slope of the calibration curve is more reproducible than the intercept, and the slope directly correlates with PCR efficiency. Hence, only a single standard point is required to “re-register” a previously performed calibration curve level for the new unknown samples. However, this assumes that the efficiency in a given run is the same as in a previous run.
8. Amplify a standard curve as described in the Subheading 3.9. In this case, the reference template *has to be the same* for both primer sets, and preferably one of the cDNA samples which is going to be used for the subsequent experiment.
  - After having set the threshold (0.1), export the Ct values to EXCEL and average the Ct of replicate samples.
  - Calculate the  $\text{LOG}_{10}$  of the given arbitrary concentration (LOGconc).
  - Obtain the  $\Delta\text{Ct}$ : for each dilution point, calculate the difference between the  $\text{Ct}_{(\text{RG})}$  and  $\text{Ct}_{(\text{GOI})}$ . Plot the LOGconc vs.  $\Delta\text{Ct}$  and obtain the equation of the curve.

If the efficiencies of the two primer sets are approximately equal, the obtained curve should be a nearly horizontal line with a slope  $< \pm 0.1$ . If this is the case, the experiment can be analyzed with the  $\Delta\Delta C_t$  method.

## References

1. Nolan, T., Hands, R.E., Bustin, S.A. (2006) Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* **1**: 1559–1582.
2. Bustin, S.A., Benes, V., Nolan, T., Pfaffl, M.W. (2005) Quantitative real-time RT-PCR - a perspective. *J. Mol. Endocrinol.* **34**: 597–601.
3. Pfaffl, M.W., Hageleit, M. (2001) Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. *Biotechnol. Lett.* **23**: 275–282.
4. Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**: e45.
5. 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**: RESEARCH0034.
6. Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P. (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**: 509–515.
7. Latorra, D., Arar, K., Hurley, J.M. (2003) Design considerations and effects of LNA in PCR primers. *Mol. Cell. Probes* **17**: 253–259.
8. Latorra, D., Campbell, K., Wolter, A., Hurley, J.M. (2003) Enhanced allele-specific PCR discrimination in SNP genotyping using 3' locked nucleic acid (LNA) primers. *Hum. Mutat.* **22**: 79–85.
9. Livak, K.J., Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $[\Delta\Delta]CT$  Method. *Methods* **25**: 402–408.
10. Pfaffl, M.W., Horgan, G.W., Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**: e36.
11. Liu, W., Saint, D.A. (2002) A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal. Biochem.* **302**: 52–59.
12. Ramakers, C., Ruijter, J.M., Deprez, R.H., Moorman, A.F. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**: 62–66.
13. Tichopad, A., Dilger, M., Schwarz, G., Pfaffl, M.W. (2003) Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic Acids Res.* **31**: e122.
14. Forlenza, M., de Carvalho Dias, J.D., Vesely, T., Pokorova, D., Savelkoul, H.F., Wiegertjes, G.F. (2008) Transcription of signal-3 cytokines, IL-12 and IFN alpha beta, coincides with the timing of CD8 alpha beta up-regulation during viral infection of common carp (*Cyprinus carpio* L.). *Mol. Immunol.* **45**: 1531–1547.
15. Fleige, S., Pfaffl, M.W. (2006) RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Asp. Med.* **27**: 126–139.



<http://www.springer.com/978-1-61779-438-4>

Cytokine Protocols

De Ley, M. (Ed.)

2012, XI, 241 p., Hardcover

ISBN: 978-1-61779-438-4

A product of Humana Press