TRANSCRIPTS QUANTIFICATION BY USING IN-HOUSE MADE RT-qPCR STANDARDS

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Abstract: Quantitative Real-Time PCR is the most widely used technique for detecting and quantifying nucleic acids in biological samples. It is the most sensitive method for the detection and quantitation of gene expression levels. Two general methods for the quantitative detection of the amplicon have been established: gene-specific fluorescent probes or specific double strand DNA binding agents. The levels of expressed genes may be measured by absolute or relative quantitative Real-Time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve. Calibration curves must be highly reproducible and allow the generation of highly specific, sensitive and reproducible data. We imagined a simple, reliable, easy-to-make method for generating standard for absolute Real-Time quantification, using only common laboratory equipment and reagents. Our method is based on re-amplification of purified amplicons, therefore assuring specificity and reproducibility of the reactions.

INTRODUCTION

Quantitative Real-Time PCR is nowadays standard practice in detecting and quantifying nucleic acids in all biological samples. As a common variant, reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of mRNA. Real-Time RT-qPCR is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range [Orlando et al., 1998; Lockey et al., 1998; Bustin et al., 2000]. Theoretically, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. In practice, though, it is a common experience for replicate reactions to yield different amounts of PCR product. The development of real-time quantitative PCR has eliminated the variability traditionally associated with quantitative PCR, thus allowing the routine and reliable quantitation of PCR products. The first practical qPCR technology was the 5’-nuclase assay established in 1993, which combined the exponential PCR amplification of a specific transcript with the monitoring of newly synthesized DNA in each performed cycle [Higuchi et al., 1993; Heid et al., 1996; Gibson et al., 1996]. It is the most sensitive method for the detection and quantitation of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue samples and for the elucidation of small changes in mRNA expression levels [Pfaffl et al., 2001; Pfaffl, 2000].

Fidelity of Real-Time RT-PCR is associated with its “true” specificity, sensitivity, reproducibility and robustness and, as a fully reliable quantitative method, it suffers from the problems inherent in RT and PCR, as amplification of unspecific products, primer-dimers, amplification efficiencies, heteroduplex formation, etc. [Freeman et al., 1999]. A most important point in succeed a RT-qPCR is to choose the right detection chemistry. Two general methods for the quantitative detection of the amplicon have been established: gene-specific fluorescent probes or specific double strand DNA binding agents [Ginzinger et al., 2002]. The best-known probe-based system is TaqMan™, which makes use of the 5’-3’ exonuclease activity of the Taq polymerase to quantitate target sequences in the samples. Probe hydrolysis separates fluorophore and quencher and results in an increased fluorescence signal [Livak, 2001]. The alternative is a non-sequence specific fluorescent intercalating dsDNA binding dye, as for exemple SYBR Green I or even ethidium bromide. For single PCR product reactions with well-designed primers, SYBR Green I can work extremely well, with spurious non-specific background only showing up in very late cycles [Zipper et al., 2004]. Among the Real-Time detection chemistry, SYBR Green I and TaqMan™ assays produce comparably dynamic range and sensitivity, while SYBR Green I detection can be more precise than the TaqMan™ probe detection if reaction specificity is perfected [Schmittgen et al., 2000].

The quantification strategy is the principal marker in gene quantification. Generally two strategies can be performed in Real-Time RT-PCR. The levels of expressed genes may be measured by absolute or relative quantitative Real-Time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA expression levels. The reliability of an absolute real-time RT-PCR assay depends on the condition of ”identical” amplification efficiencies for both the native target and the calibration curve in RT reaction and in following kinetic PCR [Souaze et al., 1996]. Relative quantification is easier to perform than absolute quantification because a calibration curve is not necessary. It is based on the expression levels of a target gene versus a housekeeping gene (reference or control gene) and in theory is adequate for most purposes to investigate physiological changes in gene expression levels. The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple Real-Time RT-PCR experiments [Orlando et al., 1998]. Under certain

circumstances, absolute quantification models can also be normalized using suitable and unregulated references or housekeeping genes.

In absolute quantification, samples are compared to a standard curve obtained with known concentrations by progressive dilutions. Calibration curves must be highly reproducible and allow the generation of highly specific, sensitive and reproducible data [Bustin et al., 2000; Pfaffl et al., 2001]. The external calibration curve model has to be thoroughly validated as the accuracy of absolute quantification in Real-Time PCR depends entirely on the accuracy of the standards. Standard design, production, determination of the exact standard concentration, and stability over long storage is not straightforward and can be problematic. The dynamic range of the performed calibration curve can be up to nine orders of magnitude from $<10^1$ to $>10^{10}$ start molecules, depending on the applied standard material [Pfaffl et al., 2001]. The calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules, as recombinant plasmid DNA, genomic DNA, RT-PCR products or commercially synthesized big oligonucleotides [Reischl et al. 1995]. Plasmid DNA or *in vitro* transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A_{260} and converted to the number of copies using the molecular weight of the DNA or RNA. Stability and reproducibility in qPCR depends on the type of standard used and depends strongly on good laboratory practice. The following critical points must be considered for the proper use of absolute standard curves:

- The standard DNA or RNA must be a single, pure species. For example, plasmid DNA is often contaminated with RNA, increasing the A_{260} value and inflating the copy number determined for the plasmid.
- Accurate pipetting is required because the standard must be diluted over several orders of magnitude. Plasmid DNA or *in vitro* transcribed RNA must be concentrated to measure an accurate A_{260} value. This concentrated DNA or RNA must be diluted up to 1000 fold to be at a concentration similar to the target in biological samples.
- The stability of the diluted standards must be considered, especially for RNA. Standards should be diluted into small aliquots, stored at −80ºC, and thaw only once before use [Collins et al., 1995].
- Generally, it is not recommended to use DNA as a standard for absolute quantitation of RNA because there is no control for the efficiency of the reverse transcription step.

We imagined a simple, reliable, easy-to-make method for generating standard for absolute Real-Time quantification, using only common laboratory equipment and reagents. Our method is based on re-amplification of purified amplicons, therefore assuring specificity and reproducibility of the reactions.

MATERIAL AND METHODS

Total RNA was extracted comparatively from either solid tissues or cultured cells, using commercial available kits (SV® Total RNA Isolation System, *Promega*™, Genelute® Mammalian Total RNA Miniprep Kit, *Sigma-Aldrich*™, and TRIZOL® Reagent, *Invitrogen*™). Kits were compared regarding quantity, purity and integrity of extracted RNA (data not shown). A DN-ase treatment step was added to all extraction protocols. RNA was rehydrated in nuclease-free H_2O, imediately retrotranscribed into cDNA, or aliquoted and kept at -80C until use. RNA quantity was estimated by spectrophotometry, measuring absorbance at 260 nm, the ratio 260/280 and with background correction at 320 nm. RNA integrity was estimated by electrophoresis in agarose TAE gel stained with ethidium bromide, in order to visualise clear 18s and 28s rRNA bands. 1 to 5 µl RNA were reverse transcribed to cDNA, either using ImProm-II® Reverse Transcription System, *Promega*™, or the M-MLV Reverse Transcriptase, *Sigma-Aldrich*™. Random hexamers or oligo-dT primers were used for RT reactions. cDNA was stored at 4°C for immediate amplifications or at -20°C for further applications. Standard curves for absolute quantification in Real-Time PCR were obtained as described below.

RESULTS AND DISCUSSION

Here we describe our in-house method for generating standard curves for interferon-gamma (IFN-γ) gene of interest and for beta-actin (β-actin) reference gene. Both curves were used in a SybrGreen I detection system and we strongly believe our system can be optimized and be functional for any interest or reference gene. For comparison, an example of TaqMan™ detection system with standard curve for GAPDH reference gene is also presented.

The first step is to generate pure amplicons from cDNA. Specific primers were designed for amplifying IFN-γ, β-actin or GAPDH genes. Primers were designed to span exon-exon junctions only available for hybridization in cDNA, thus avoiding any cross-amplification of undigested genomic DNA. We used the following primers:

- For IFN-γ : (437 bp amplicon)

Forward : GGCTGTTACTGCCAGGACCCATATGT
Reverse : GATGCTCTTCGACCTCGAAACAGCAT

- For β-actin (661bp amplicon)

Forward : TGACGGGGTCACCCACACTGTGCCCATCTA
Reverse : CTAGAAGCATTTGCGGTGGACGATGGAGGG

- For GAPDH (349 bp amplicon)

Forward ATCATCCCTGCCTCTACTGG
Reverse : TGCTGTAGCCAAATTCGTTG

In all cases, amplification was performed in a final volume of 50 µl containing 5 µl cDNA, 1,25 units of GoTaq® Flexi DNA Polymerase (*Promega*™) in apropriate 1X buffer, 1,5mM $MgCl_2$, 0,4 µM each primer, and 0.2mM each dNTP. Cycling program was composed of an initial denaturation of 15 min 95ºC, followed by 45 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min, plus a final extension of 10 minutes at 72ºC. This common PCR program allows amplification of all three amplicons.

In the case of IFN-γ and GAPDH genes, unspecific products may appear when using these cycling conditions, which is not the case for the β-actin. For detecting such events, 20 µl of each PCR reaction are migrated in a 1X TBE agarose gel, for 30 minutes at 7V/migration distance. Visualizing gels stained with ethidium bromide allows identification of specific amplicons and unspecific products. When only a specific band is observed (case of β-actin), the amplicon can be purified from the remaining 30 µl PCR product, by using the Wizard® PCR Preps DNA Purification System (*Promega*™), following producer's indications for "purifying PCR products directly from PCR reactions". When unspecific products are either observed in migration profile, the specific band is cut from the gel under UV, and specific amplicon is purified from the gel using the Wizard® PCR Preps DNA Purification System (*Promega*™), following producer's indications for "purifying PCR products from agarose gels". In both cases, the amplicon DNA is eluted in 50 µl H_2O. Its concentration can be measured by spectrophotometry at 260 nm, which was nor possible for cDNA.

Knowing the size of the amplicon in base pairs and its concentration in ng/µl, any easy internet available convertor, such as the *Promega*'s Math calculator, can be used to calculate the molarity of amplicon solution obtained. Molarity can be used to estimate the number of molecules present in a given volume of the same solution, as is presented in Table I.

Table I. Estimating the number of molecules in solution for each amplicon

Amplicon	DO_{260}	DO_{280}	DO_{260}/DO_{280}	Concentration (ng/µl)	Size (bp)	Molarity (pmol/ µl)	Molecules / µl
IFN-γ	0,062	0,035	1,775	3,1	437	10,63	$6,4 * 10^9$
GAPDH	0,173	0,101	1,714	8,65	349	37,66	$2,3 \times 10^{10}$
β-actine	0,376	0,223	1,684	7,52	661	17,191	10^{10}

The last operation to do is to dilute each amplicon solution progressively (10 X dilutions, 10 µl with 90 µl H_2O), in order to obtain a known series of concentrations (molecules). Even if the estimations are slightly similar for each amplicon in Table 1, the range of dilutions used for Real-Time PCR was different in each case, depending on the chemistry and the efficiency of each reaction. Briefly, 5 µl purified amplicon (from the concentrated solution, or from each dilution obtained) served as template in a Real-Time PCR amplification, using the same forward and reverse primers as shown before, and using a SybrGreen I detection system based on the SensiMixPlus® SYBR Kit, *Qantance*™, following producer's instuctions.

In figure 1 is presented the quantitative analysis for IFN-γ standards amplification, ranged from 50 to 6400 template molecules. It is a very good standard curve able to quantify little

quantities of IFN-γ mRNA as it really happens *in vivo*. Figure 2 shows a melting curve obtained from the same amplified products, which demonstrate the specificity of the Real-TIme PCR reaction. However, one may easily observe below 400 template molecules (standard sample no. 5) the apparition in the melting curve of a noise, left-sided from the main specific product (Tm = 85ºC). This corresponds to a primer dimer appearing when not enough template is available. Negligible at the beginning, this unwanted product becomes intrusive for our last standard sample (no. 8). Therefore we cannot recommend using standards below 50 molecules copies, as primer dimer background could false the fidelity of the standard curve and of the quantification.

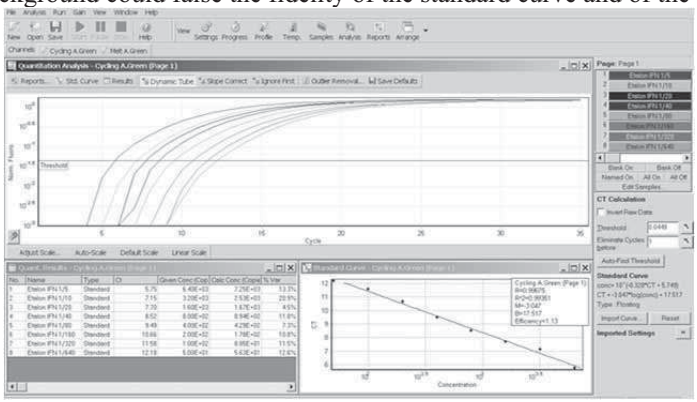

Figure 1. Quantitation analysis for a standard curve generated for IFN-γ cDNA.

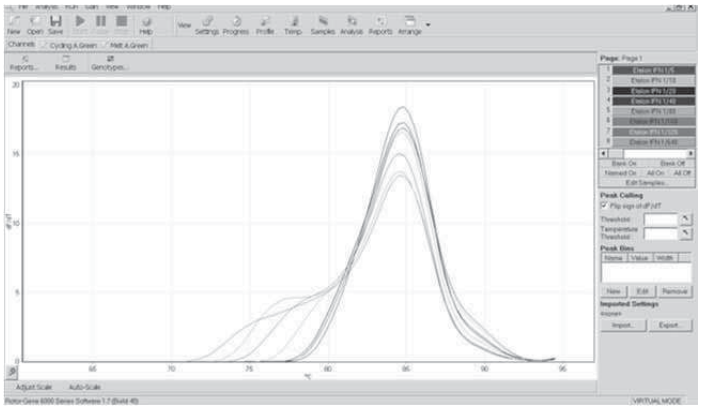

Figure 2. Melting analysis for a standard curve generated for IFN-γ cDNA.

In figure 3, we show the example of analysis obtained for the β-actin standard curve, with samples ranged from 525 to 1.050.000.000 molecules. The standard curve is, here again, large-ranged, reliable and precise. For standards below 500 molecules, the signal was less intense while the specificity of the reaction remained stable. Contrarily, another problem appears for standards containing more than 100.000 molecules. We can observe, in the melting curve, the apparition of a right-sided unspecific product when template is too abundant, as it really happens *in vivo*. Therefore, we do not recommend using standards with more than 100.000 copies, but rather to dilute samples in order to frame them below this value.

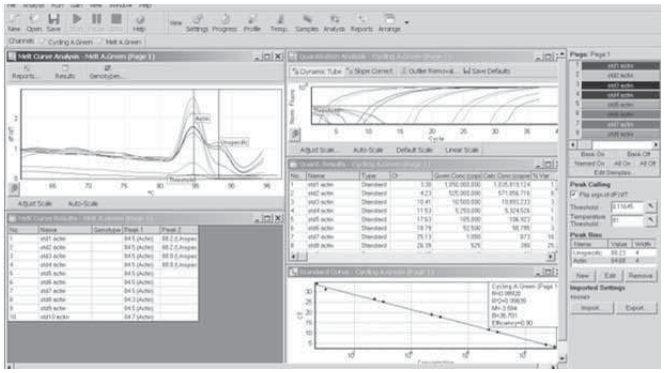

Figure 3. Quantitation and melting analysis for a standard curve generated for β-actin cDNA.

In figure 4, we present a good standard curve generated for GAPDH gene cDNA, with dilutions ranged from 25 to 1.000.000 molecules. Since the detection system used for GAPDH was TaqMan™, melting curve was not possible, although TaqMan™ system ensures by itself the specificity of the Real-Time amplification. Therefore, we do recommend the usage of the curve for the interval mentioned.

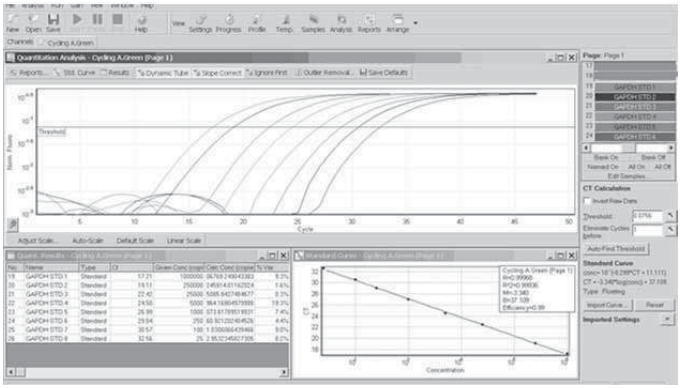

Figure 4. Quantitation analysis for a standard curve generated for GAPDH cDNA.

DISCUSSION

Quantitative Real-Time PCR is extremely useful for measuring gene expression, viral charge, external DNA contaminants, and generally for determining nucleic acid quantities. Absolute and relative quantifications can be used for such approaches. Absolute quantitations always need an external standard curve of known progressive DNA or RNA concentrations. Several approaches were used in time for generating either RNA or DNA standard curves. We presented an in-house, rapid and efficient method for obtaining cDNA standard curves for interest and reference genes, applicable for Sybr Green or TaqMan™ analysis. Our method was validated for IFN-γ, β-actin and GAPDH genes, and we showed the confidence intervals for each concentration ranges.

Certainly, a problem with DNA-based calibration curves is that they are subject to 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 unknown samples. However, the problem of the

sensitivity of the RT-PCR to small variations in the reaction setup is always lurking in the background as a potential drawback to this simple procedure. Therefore, quantification with external standards requires careful optimization of its precision and reproducibility (intra-assay and inter-assay variation), in order to understand and assume the limitations for each application.

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