Real-Time PCR Quality Control for Gene Expression Profiling Using the LightCycler® 480 System

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Introduction

Quantitative real-time PCR (qPCR) has become the *de facto* standard for nucleic acid quantification. This achievement is due in large part to its sensitivity, specificity, accuracy, large dynamic range of linear quantification, and its speed. The qPCR technology has matured to a ready-to-use commonly available method in most molecular biology laboratories. Nevertheless, the reliability of the final quantification result depends heavily on all elements in the workflow, such as the quality of the input template (integrity and absence of inhibitors), the PCR assay (specificity, efficiency, limit of detection), and normalization strategy (validated reference genes).

Materials and Methods

As an integral part of any qPCR experiment, we perform different quality control studies during the workflow. For gene expression measurements for example, we first determine the quality of the input RNA by assessing the presence of putative inhibitors using the recently described SPUD assay [1], in which a synthetic oligonucleotide is amplified in the presence or absence of RNA. Only when the Cq values (quantification cycle value, universal term according to the real-time PCR data mark-up language RDML, www.medgen.ugent.be/rdml) are within 0.5 cycle difference do we assume that there are no major inhibitors. To assess the integrity of the total RNA, the RNA samples are tested on a capillary gel electrophoresis instrument, such as the Bioanalyzer (Agilent Technologies) or Experion (Bio-Rad Laboratories), whereby either an RNA integrity number (RIN) or an RNA degradation factor is calculated. We have recently begun to employ a PCR-based assay to measure mRNA integrity. In this assay, the ratio of the 5' versus the 3' end of a well-known and abundant reference gene (*e.g.*, *GAPDH*) is quantified in the samples of interest and compared with an intact reference sample or standard series of equimolar dilutions [2]. After establishing the quality of the RNA, a proper DNase treatment in solution is performed and then verified by performing a DNA-targeted qPCR on the DNase-treated RNA, whereby no signal should be observed.

A second level of quality control in the experimental workflow is assessing the actual qPCR assay. Prior to ordering the forward and the reverse primer and probe(s), we test four quality issues using our own established and freely available automated *in silico* assay evaluation pipeline (www.medgen.ugent.be/rtprimerdb) [3]. Provided with basic information such as the organism of interest, the official gene symbol, the primer sequences, the template (cDNA or DNA), and the intended annealing temperature, RTPrimerDB determines if there are any known SNPs contained in the oligonucleotide sequences, initiates a BLAST specificity search and an MFOLD secondary structure analysis, and determines the specificity of the assay with respect to known transcript variants. Following the *in silico* evaluation of the assay, the efficiency and sensitivity (limit of detection) is experimentally tested using a serial dilution of a commercially available qPCR reference total RNA (see Figure 1 for an

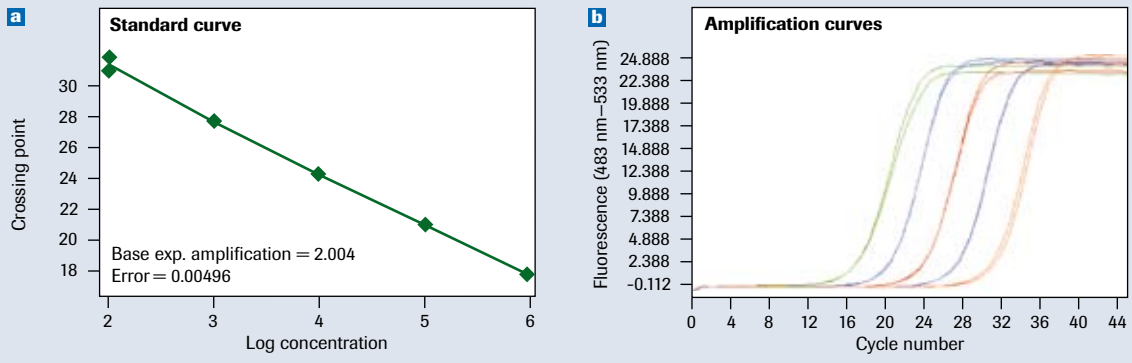

Figure 1: Tenfold serial dilution (10^6–10^2 molecules) standard curve demonstrating a well-performing SPUD assay.

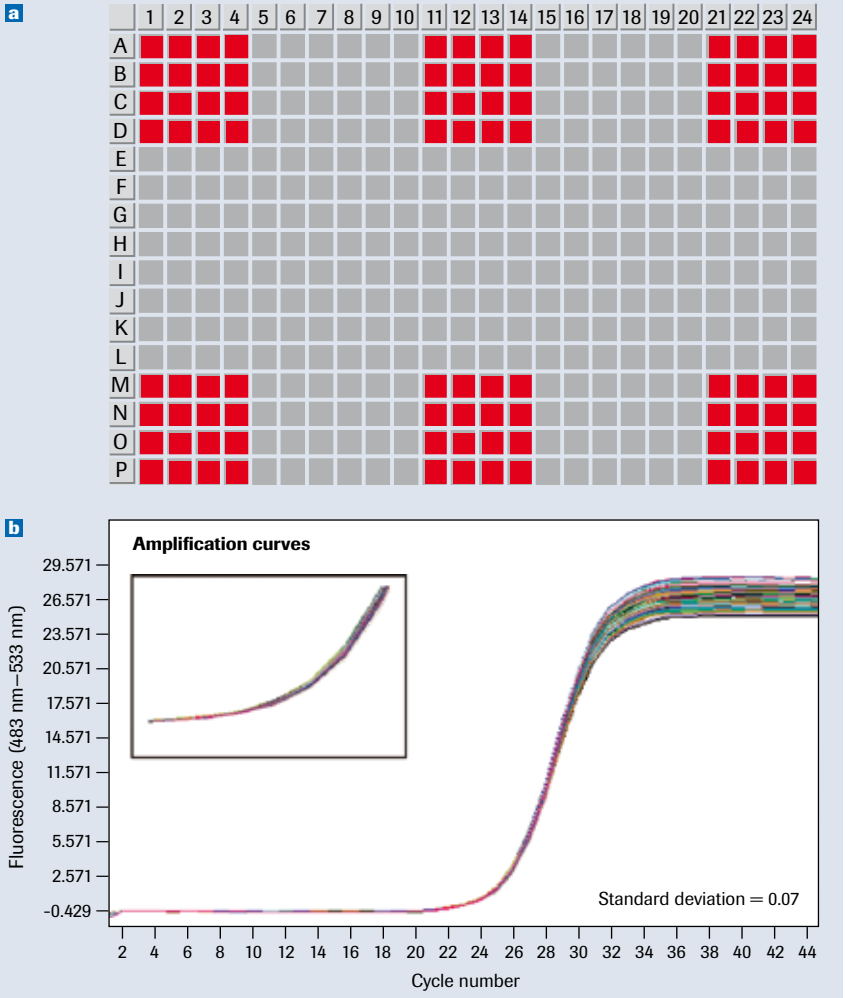

Figure 2: (a) Block layout and (b) resulting amplification plots of 96 replicated PCR reactions (5 ng DNA) using the LightCycler® 480 Instrument.

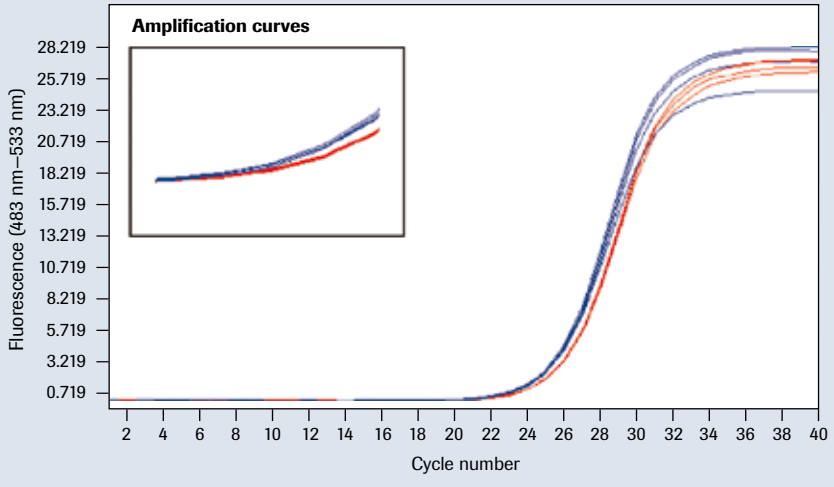

Figure 3: Superior reproducibility allowing 1.33-fold discrimination between 3,000 copies (10 ng DNA; blue) and 2,250 copies (7.5 ng DNA; red).

example of a good assay, with almost perfect efficiency, low error, and large dynamic range of linear quantification). The protocol for preparing such a standard curve (in a background of carrier DNA to minimize adsorption and Poisson effects) can be found on our website (www.medgen.ugent.be/CMGG/protocols, see "Template dilution series for qPCR assay evaluation").

An often underestimated source of variation is the real-time PCR instrument itself. Apart from template and assay quality control, the performance of the instrument can also impact the quantification result. To assess the homogeneity of results across a block-based cycler, we regularly apply a so-called homogeneity assay to ensure optimal performance over time. By comparing these results with benchmark data generated from different block-based instruments during their first usage, the temperature uniformity of the block and the homogeneity of the optical system is inspected. For this purpose, a huge master mix containing all components to perform a PCR (*i.e.*, DNA polymerase, buffer, dNTPs, primers, and template) is prepared and distributed using a repetition pipette in 96 wells of either a 96- or a 384-well block. This instrument verification experiment perfectly captures any inconsistencies that arise across the block. By recording the Cq values and calculating the standard deviation, inter-quartile range (75^{th} percentile minus 25^{th} percentile Cq value), and maximal Cq difference across the block, a good estimate of block homogeneity and technical reproducibility of a well-performing qPCR instrument is obtained. Figure 2 shows the block layout and tight amplification plots of 96 replicated reactions spread across the LightCycler® 480 384-well block.

To further explore the LightCycler® 480 384-well block performance, we designed a resolution determination experiment, in which the minimum-fold difference in copy numbers that could be reliably detected using quadruplicated PCR reactions was investigated. Figure 3 clearly demonstrates that a 1.33-fold difference in molecules can be reliably detected ($p<0.05$). Important to note is that this result does not mean that a 1.33-fold difference in gene expression can be reliably measured. For this kind of experiment, normalization is of crucial importance, whereby well-validated reference genes must be used. However, we and others have shown that careful experiment design and the use of multiple stably expressed reference genes can result in the reliable detection of a twofold expression difference (see for example reference [4]).

For all above-described experiments using the LightCycler® 480 System, we used the LightCycler® 480 SYBR Green I Master in a total volume of 8 µl on a 384-well

plate, with the following temperature protocol: 5 minutes 95°C polymerase activation hot start, followed by 45 cycles of 10 seconds 95°C, 30 seconds 60°C, 1 second 72°C (data collection step), and a melting curve analysis (from 60°C to 95°C). The primers for Figure 1 are reported in reference [1], the primers for Figure 2 and 3 are reported in reference [5] and available in the primer and probe database RTPrimerDB (medgen.ugent.be/rtprimerdb) under ID 1024. All primers are used at a final concentration of 250 nM. For Cq value determination, we used the second derivative maximum method in the LightCycler® 480 quantification software, exported data to Microsoft Excel and calculated the reproducibility parameters.

Table 1: Descriptive statistics for PCR replicates using the LightCycler® 480 Instrument and two other 96-well block instruments.

	LightCycler® 480 Instrument		Instrument A	Instrument B
	Repetition pipette[a] 384-well	Robot[b] 384-well	Repetition pipette[a] 96-well	Repetition pipette[a] 96-well
Replicates	96	96	96	96
Standard deviation	0.070	0.095	0.159	0.170
IQ	0.07	0.11	0.22	0.19
maximal Cq difference	0.41	0.46	0.65	1.00

[a] PCR mix including DNA template
[b] Separate pipetting of PCR mix and DNA template
IQ: Inter-quartile range (75th percentile minus 25th percentile Cq value [*i.e.,* the maximal Cq value difference among the 50% best replicates])

Results and Applications

In Table 1, descriptive statistics for representative block homogeneity experiments using the LightCycler® 480 Instrument (384-well block) and two other 96-well block instruments A and B are summarized. These data clearly demonstrate a very high reproducibility of the LightCycler® 480 384-well block. We recently obtained similar outstanding values using a well-trained pipetting robot distributing 3 µl template (5 ng human genomic DNA) to 5 µl master mix (using a Caliper ALH3000 with an 8-channel master mix dispensing head and 96-needle template pipetting capacity).

The high reproducibility of replicated reactions using the LightCycler® 480 System certainly contributes to the fact that a 1.33-fold difference (equivalent to a 33% increase or a 25% decrease) in molecules can be reliably detected. This opens perspectives to applications in which subtle differences in nucleic acids must be detected (*e.g.*, single exon deletions in human disease, or gene silencing through RNA interference). Furthermore, this allows the use of innovative and powerful methods such as high-resolution melting curve analysis, which demand minimal differences across the block. We are currently evaluating this technology using the LightCycler® 480 Instrument, with successful preliminary results.

Conclusions

In conclusion, several elements of the qPCR gene expression workflow must be well-controlled, including block homogeneity, to achieve reliable quantification with high accuracy and precision. The LightCycler® 480 Instrument with its 384-well block has superior block performance with respect to reproducibility of replicated PCR reactions. This might prove very useful when assessing small differences in gene expression or copy number. ■

References

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Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	1 instrument (384 well)	04 545 885 001
LightCycler® 480 Multiwell Plate 384	5 x 10 plates (includes sealing foils)	04 729 749 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 SYBR Green I Master	5 x 1 ml (2x conc.) (approx. 500 reactions of 20 µl final reaction volume)	04 707 516 001
LightCycler® 480 SYBR Green I Master	10 x 5 ml (2x conc.) (approx. 10 x 500 reactions of 20 µl final reaction volume)	04 887 352 001