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APPLICATION NOTE No. 226

Use of LoBind® Tubes to Consistently Prepare and Store Standard Panels for Real-time PCR Absolute Quantifications

Maxime Lecerf, Jérôme Le Goff*
Université Paris V, Equipe "Immunité et Biothérapie Muqueuses", Unité INSERM Internationale U743 (Immunologie Humaine), Centre de Recherches Biomédicales des Cordeliers, 15 rue de l'Ecole de Médecine, 75270 Cedex 06, Paris, France
*Corresponding author e-mail address: maxime.lecerf@crc.jussieu.fr

Abstract

This Application Note describes how the choice of vessel can affect the reliability of DNA standard panels preparation used in real-time PCR assays for absolute quantification. Results underscore nucleic acids retention on different tubes used for serial dilution and storage of standards. Consequently, apparent amplification efficiencies were up to 30 percentage points lower with competitor low retention tubes than with Eppendorf DNA LoBind® tubes.
Incorrect standard curves obtained from panels prepared in such competitor's tube can lead to misinterpretation of PCR efficiency values and to significant overestimation of DNA quantity in samples assayed.

Introduction

Real-time PCR is a powerful technology for quantification of target nucleic acids sequences as it combines specificity and sensitivity. The so-called absolute quantification enables to determine DNA concentrations of samples by running standards in parallel. However, this requires strict setup of the PCR reaction and rigorous standard panel preparation in order to obtain reliable results [1]. Although primers and probe design, as well as PCR condition setup are often the trickiest steps, standards preparation must not be underestimated. Standard panels serve as a basis for calibration curve by plotting C_t values of the standards against $\log_{10}$ of corresponding amounts of DNA.

The slope of this curve reflects the amplification efficiency and can be determined as follows:

Eq. 1: $Efficiency = 10^{-1/Slope} - 1$

Optimized PCR, being an exponential reaction at least in the beginning, with a theoretical maximum efficiency of 1.00 (or 100 %) means that amplicon quantity doubles at each cycle. Moreover, calibration curve also allows user to determine template concentration of samples from C_t values which were determined experimentally.

In this context, standard panel preparation appears as a crucial step in the reliable assessment of amplification efficiency and correct sample quantification. However, this preparation is often challenging because a good panel should range over several orders of magnitude of dilution (log_{10} steps) [1]. Dealing with very low concentrations of nucleic acid such as 10 copies of genomic DNA per 10 µL involves that sample adsorption on vessels cannot be ignored. Indeed, the loss of DNA on vessel surfaces will be amplified at each dilution step and will be particularly significant for the most diluted standards leading to a DNA concentration actually lower than expected. Accordingly, the observed amplification efficiency will be significantly reduced not because of a problem with the enzymatic reaction but simply because the initial DNA quantity is overestimated, leading to misinterpretation of the results.

The following experiment will describe how the choice of laboratory vessel influences the apparent real-time PCR efficiency by preparing serial dilution of standard panels and/or storing them on Eppendorf DNA LoBind or competitor low retention tube.

Material and Methods

Nucleic acid purification: Genomic DNA was obtained from lymphoblastoid T-cell line 8E5 (NIH; ATCC 8993). Cultured cells were harvested, counted under microscope and resuspended in PBS at 5×10^6 cells/mL. DNA was extracted from 200 µL of cell suspension using the QIAamp® DNA Blood Mini Kit (Qiagen, Germany) according to manufacturer's protocol. Elution was made in 200 µL of water (Biotechnology Performance Certified, Sigma-Aldrich, USA) to obtain a stock solution at 1×10^5 copies of genomic DNA per 10 µL.

Genomic DNA panel: Serial 10-fold dilutions were carried out as follows in 1.5 mL tubes (either Eppendorf DNA LoBind or competitor low retention tubes): 100 µL of genomic DNA solution was transferred in a tube containing 900 µL of water according to Eppendorf pipetting guidelines. Tube was then vortexed for one minute and served as start solution for the next dilution step. Then, each dilution was aliquotted by 22 µL either on Eppendorf DNA LoBind tubes or competitor low retention tubes and stored at -80 °C for 24 hours (Figure 1).

Quantification of genomic DNA target: Target gene (*ALB*) was quantified by real-time PCR.
The forward primer was
5'-GCTGTCATCTCTTGTGGGCTGT -3',
the reverse primer was
5'- AAACTCATGGGAGCTGCTGGTT -3'
and the probe was
5'- FAM CCTGTCATGCCCACACAAATCTCTCC TAMRA -3' [2].

Primers and probes were synthesized by Eurogentec (Belgium). Real-time PCR was carried out on a LightCycler® instrument using the LightCycler® FastStart® DNA Master HybProbe® kit (Roche Applied Science, Switzerland) [3]. 20 µL capillaries contained 1 x FastStart® DNA Master HybProbe buffer, additional 3 mM $MgCl_2$, 0.3 µM each primer and probe and 10 µL of genomic DNA standard. Thermocycling conditions consisted of an initial step (95 °C for 10 min) and 45 cycles of denaturation (95 °C for 10 sec) and an annealing and polymerization step (60 °C for 30 s). All three standard panels were assayed in the same experiment.

Standard curve: C_t values were plotted versus the log_{10}-transformed expected concentration of target gene. Linear regression was applied to each scatterplot and slope was used to determine apparent amplification efficiency using equation 1.

Results and Discussion

Figure 2 shows the standard curves obtained for each panel. Panels 1 and 2 originated from the same serial dilution carried out in competitor low retention tubes. Panel 1 was stored aliquoted in competitor tubes and panel 2 in Eppendorf DNA LoBind tubes. Panel 3 was both diluted and stored in Eppendorf DNA LoBind tubes. Apparent efficiencies were 56 % for panel 1, 69 % for panel 2 and 86 % for panel 3 and C_t values at 10^0 copies/10 µL were 41.00, 35.44 and 33.65, respectively.
As these three standard curves were obtained in the same experiment, it is highly likely that the differences observed are due to a loss of genomic DNA. This loss can occur both during dilution preparations, leading to a decrease by 17 percentage points in apparent efficiency, and during storage giving an additional 13 percentage points decrease. The C_t values are also consistent with DNA adsorption on tubes as the maximum effect is observed for lowest DNA concentration (up to 7 C_ts of difference at 10^0 copies/10 µL).

These results highlight an obvious effect of vessel quality in the accuracy of standard panel preparation. In a more general extent, they reveal how much DNA adsorption can become a major point when dealing with low concentrations. applied to each scatterplot and slope was used to determine apparent amplification efficiency using equation 1.

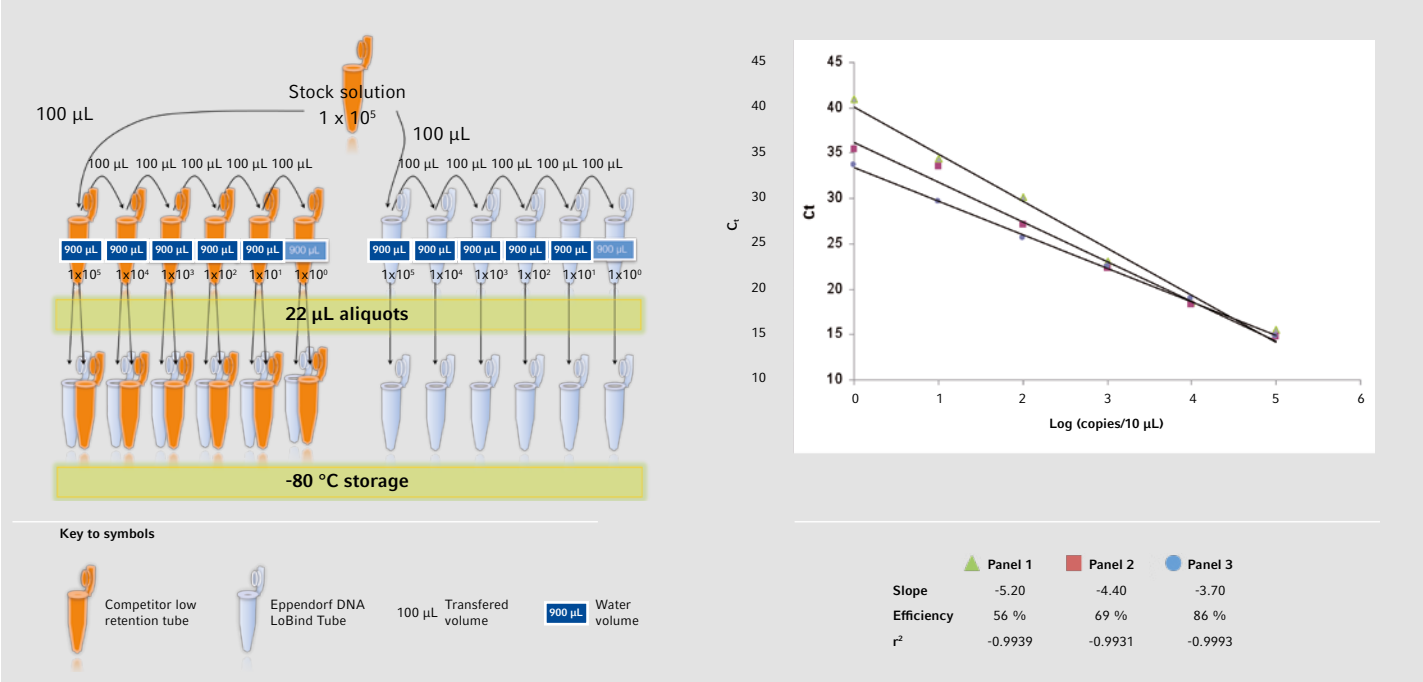

Fig. 1: Flowchart of genomic DNA standard assay panels preparation. DNA stock solution was serial 10-fold diluted in either competitor's low retention or Eppendorf DNA LoBind tubes. 22 µL aliquots of each dilution were stored in either competitor's low retention or Eppendorf DNA LoBind tubes. Aliquots were stored at -80 °C for 24 hours before real-time PCR assay.

Fig. 2: Standard curves obtained for each panel. C_t versus $\log_{10}$ of expected DNA concentration was plotted and slopes were obtained after linear regression. PCR efficiencies are derived from slope values.

Literature

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