

The MIQE Guidelines and Assessment of Nucleic Acids Prior to qPCR and RT-qPCR

Andrew F. Page

Thermo Fisher Scientific, Wilmington, DE

Introduction

Quantitative PCR has emerged as a fundamental technique in molecular & clinical diagnostics. Before now, little or no standardization in reporting existed, and as a result, experiments were frequently hard to replicate. In addition, many assumptions upon which the technique depends, in particular those pertaining to reference gene stability and amplification efficiency, were often in fact invalid. In order that experiments be better designed and appropriately validated, and that experimental reporting include sufficient information for experimental replication, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have been proposed (1). This application note examines the MIQE guidelines relevant to the assessment of nucleic acids, in particular quantification and purity assessment of nucleic acids using Thermo Scientific NanoDrop spectrophotometers.

MIQE Guidelines

The MIQE guidelines (1) are similar in principle to the MIAME guidelines used for microarray experiments. The rationale for the guidelines is that a full disclosure of methods allows assessment of protocol validity, correct interpretation of results and reproducibility. The guidelines cover all aspects of qPCR experiments, from experimental design to data analysis, although the bulk of the guidelines are associated with the experimental protocol itself.

The guidelines stipulate that nucleic acid quantification post-extraction is “Essential”, and that an assessment of purity using spectrophotometry is “Desirable”. This document explains these requirements by describing some of the potential risks of not quantifying nucleic acids before qPCR or RT-qPCR. Furthermore, we show here the value of a purity assessment; in particular how detection of contaminants can often be achieved by spectrophotometry.

Quality Control

The ability to QC samples both accurately and easily is important for any experiment. Failure to properly QC samples could result in time delays or additional costs. As molecular techniques such as qPCR continue to use smaller amounts of

sample, the quality control of these samples is essential. Conserving limited, often highly valuable samples is paramount in situations that involve extraction of samples from a limited cell mass. The microvolume capability of a Thermo Scientific NanoDrop UV-Vis Spectrophotometer allows the researcher to quickly and easily run quality control checks of nucleic acid and protein samples.

NanoDrop™ Spectrophotometers

NanoDrop spectrophotometers utilize a revolutionary sample retention technology which retains 1 µL samples in place via surface tension between two fiber optic cables. After measurement, samples are quickly and easily removed from the optical surfaces with an ordinary dry laboratory wipe.

The NanoDrop 2000/2000c spectrophotometer utilizes multiple path lengths (1.0 mm, 0.2 mm, 0.1 mm and 0.05 mm) that change in real time while measuring a 1 µL sample, resulting in a wide dynamic range capable of measuring 2 - 15,000 ng/µL of dsDNA. In contrast to this, measuring samples with a standard 10 mm quartz cuvette on a conventional spectrophotometer has an upper detection limit of 50 ng/µL with a minimum sample volume of 1 mL. Moreover, the use of cuvettes can potentially lead to cross-contamination from prior samples if not properly cleaned.

NanoDrop users also benefit from a quick measurement cycle. The total cycle time for the NanoDrop 2000c is five seconds or less, and in cases where higher throughput is required, the NanoDrop 8000 can measure up to eight samples with a total cycle time of just 20 seconds. The intuitive software displays the calculated DNA concentration, nucleic acid purity ratios, and spectra of each sample.

Why is Quantity so Important?

For absolute quantification using a standard curve, samples must lie within the range of the standard curve. When plotted as shown in fig. 1, standard curves are linear, however they do not remain so indefinitely. Once template concentrations exceed a level at which they are limiting the PCR reaction, amplification

efficiency decreases. At low template concentrations, background contamination may be mistaken for amplification signal, and data variability becomes an increasing problem. Extrapolating standard curves to encompass greater ranges is therefore a hazardous practice; it is far better to ensure before qPCR that samples lie within a suitable concentration range.

For relative quantification, e.g. gene expression by RT-qPCR, low template quantities increase error. Even methods producing qualitative data such as SNP genotyping are more reliable when performed using appropriate template quantities. This is shown in fig. 2, which illustrates that signal strength for unknown samples should be similar to that for standards. Low template quantities make allele calls unreliable or impossible.

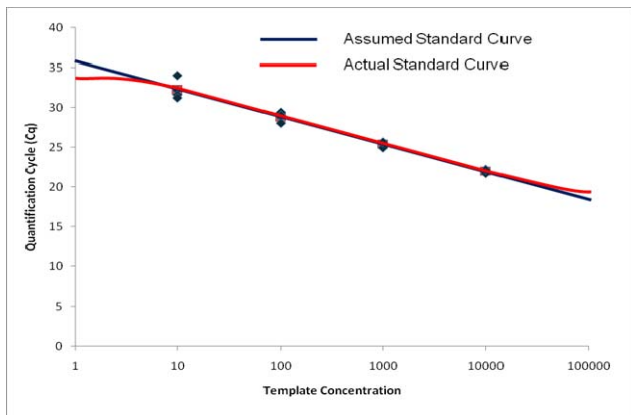


Figure 1: Extrapolation of a standard curve outside the range encompassed by the standards or adding additional standards is dangerous as standard curves do not remain linear indefinitely. Unknown samples quantified at extrapolated regions of a standard curve rely on the unproven and risky assumption that the curve is still linear at that point.

When Exact Quantification is Critical

Sometimes equal loading of different templates is an integral part of the experimental design, e.g. gene expression studies where samples are normalized to total RNA input. A more common instance where exact quantification is critical is the validation of new reference genes.

Validation of new reference genes for RT-qPCR may be accomplished by first extracting RNA from control and experimental samples. Following reverse transcription, cDNA quantities are normalized to ensure equal loading into qPCR reactions. Quantitative PCR is then performed using primers

(and probe, if relevant) for the candidate gene, and the C_q measured for each sample. Statistical analysis of C_q cycle numbers is then performed in order to test for a difference between the control and experimental samples. If no such difference exists, then the expression of the gene of interest may be concluded to be the same in the control and experimental samples. The candidate reference gene therefore is suitable for future studies of gene expression using the tested combination of control and experimental samples.

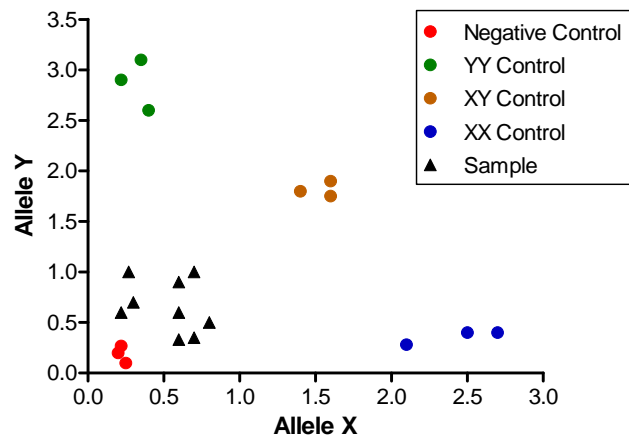
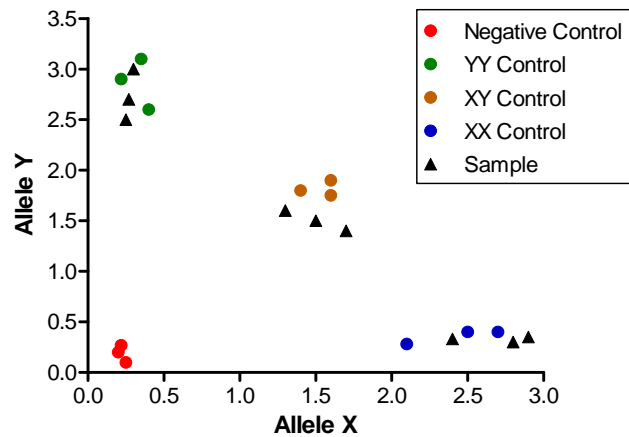
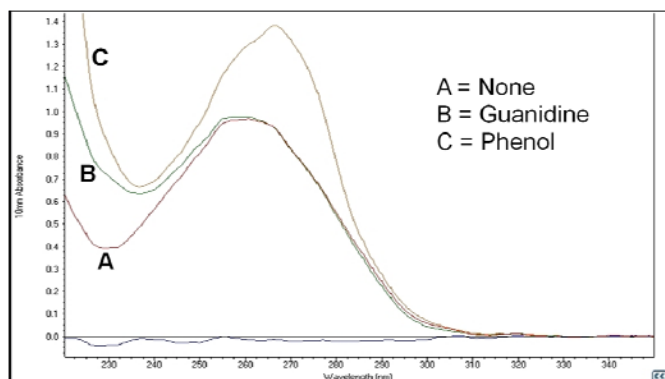


Figure 2: Example of SNP genotyping using appropriate template quantities (above) and sub-optimal template quantities (lower). Note that with appropriate template quantities, allele calls for unknown samples are clear, while low template quantities make allele calls unreliable or impossible.

Why is Purity so Important?

Residual chemical contamination from extraction procedures can drastically influence downstream analysis, however many contaminants are detectable using NanoDrop spectrophotometers. Figure 3 shows a purified DNA sample (A), along with the same sample contaminated with guanidine (B) and phenol (C). Upon initial examination, both contaminated samples appear to give a spectra characteristic of nucleic acids, and in fact the 260/280 ratios seen for phenol contaminated samples are often normal.

Figure 3: Spectra of purified DNA without contamination (A), and of the same DNA sample contaminated with guanidine (B) and phenol (C). Note the changes in wavelength of the trough and peak in the spectra, normally observed at 230 nm and 260 nm, respectively.



Although the spectra cannot diagnose problems *per se*, examination may assist in identifying that a problem exists and in narrowing down the source of the problem. Many contaminants absorb at around 230 nm or less; many of these create problems with downstream applications. In addition to examining the 260/280 ratio and the general shape of the spectra, we recommend the following:

- Check the 260/230 ratio – a poor ratio may be the result of a contaminant absorbing at 230 nm or less.
- Check the wavelength of the trough in the spectra – this should be at 230 nm. Absorbance by a contaminant at a low wavelength will typically increase the wavelength of the trough.
- Check the wavelength of the peak in the spectra – this should be at 260 nm for DNA and RNA. Absorbance at a higher wavelength by a contaminant will increase the wavelength of the peak.

Some contaminants have characteristic profiles, e.g. phenol, however many contaminants present similar characteristics: absorbance at 230 nm or less. Absorbance at 230 nm may indicate a problem with the sample or with the extraction procedure, so it is important to consider both. For example: a high A230 (poor A260/A230 ratio) may be caused by:

- Carbohydrate carryover (often a problem with plants).
- Residual phenol from nucleic acid extraction.
- Residual guanidine (often used in column based kits).
- Glycogen used for precipitation.

Conclusions

The MIQE guidelines stipulate that a measurement of nucleic acid quantity is essential, while an assessment of purity is desirable. In reality, the measurement of both quantity and purity is beneficial not only in order to design better experiments and ensure better reporting. The use of a NanoDrop spectrophotometer to examine nucleic acids can result in significant savings in time and money spent re-running samples. Re-extraction or cleanup are small delays compared to re-running an entire experiment, especially if it is necessary to delay until a critical sample number is reached.

References

- 1) Bustin SA, Benes V, Garson JA, Hellems J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 55 (4): 611-22.