Application note

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

NanoDrop Spectrophotometers

MIQE Guidelines

Quantitative PCR (qPCR) is a fundamental quantification technique used in molecular and clinical diagnostics. Before 2009, inadequate standardization in reporting existed, and as a result, experiments were frequently hard to replicate. In addition, many assumptions about the qPCR technique were often unjustified, including those pertaining to amplification efficiency and reference gene stability. To improve experimental design, validation, and reporting, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have been proposed.[1]

The rationale for the MIQE guidelines is that a full disclosure of methods allows assessment of protocol validity, appropriate interpretation of results, and consistency between laboratories. The guidelines stipulate the following as “essential” to provide in a PCR publication: nucleic acid quantification, contamination assessment (DNA or RNA), and amount of RNA/cDNA/DNA added to the reaction. It is also “desirable” to include nucleic acid purity as estimated by the A260/A280 absorbance ratio.

Quality Control with NanoDrop Spectrophotometers

Thermo Scientific™ NanoDrop™ Microvolume UV-Vis Spectrophotometers use a pioneering sample retention technology, which retains 1.0 - 2.0 µL of samples between two fiber optic cables via surface tension. This microvolume capability conserves limited, highly valuable samples for downstream molecular techniques. Outlined in Figure 1, the NanoDrop instruments utilize multiple pathlengths that change in real time during a sample measurement, resulting in a wide dynamic range.

	NanoDrop Lite Plus	NanoDrop One/One[c]	NanoDrop Eight
			
Pathlengths	1.0 mm, 0.2 mm	1.0 mm, 0.2 mm, 0.1 mm, 0.05 mm, 0.03 mm	1.0 mm, 0.2 mm, 0.1 mm
Concentration Range (dsDNA)	2.0 - 1,500 ng/µL	2.0 - 27,500 ng/µL	2.0 - 10,000 ng/µL

Figure 1. Pathlengths and dsDNA concentration ranges for the different NanoDrop spectrophotometer models. Figure created with BioRender.com.

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In contrast to the NanoDrop instruments, measuring samples with a standard 10 mm cuvette on a conventional spectrophotometer has an upper detection limit of 50 ng/µL with a minimum sample volume of 1.0 mL. This detection limit requires error-prone dilutions and a high volume of sample, limiting what can be used for downstream experiments. The use of cuvettes can also potentially lead to cross-contamination from prior samples if not properly cleaned.

NanoDrop spectrophotometers also have a quick measurement cycle. The total cycle time for the NanoDrop instruments range from 5 seconds to 20 seconds, depending on the model. The NanoDrop Eight instrument, which allows simultaneous measurement of 8 microvolume samples for higher throughput, has a total cycle time of 20 seconds or less. Intuitive NanoDrop software displays the calculated DNA concentration, nucleic acid purity ratios, and spectra of each sample. (Sample spectra are not available for the NanoDrop Lite Plus).

Why is Quantification Important?

For absolute quantification using a standard curve, samples must lie within the range of the standard curve. When plotted as shown in Figure 2, standard curves are linear, however they do not remain so indefinitely. Once template concentrations exceed a level at which they limit the PCR reaction, amplification efficiency decreases. At low template concentrations, background signals may be mistaken for amplification signals, increasing data variability. At high template concentrations, the PCR reaction may be inhibited.[2] Extrapolating standard curves to encompass greater ranges is risky; it is suggested to ensure unknown samples lie within a suitable concentration range prior to PCR.

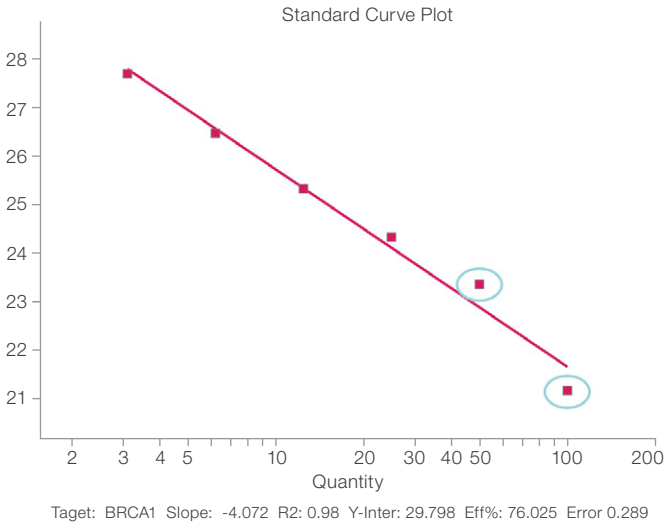

Figure 2. Example of a standard curve containing additional standards that lie outside the linear range (circled in blue). Unknown samples quantified at extrapolated regions of a standard curve rely on the unproven and risky assumption that the curve is still linear at that point.

For relative quantification, such as gene expression by RT-qPCR, low template quantities increase error.[3] Even methods producing qualitive data such as SNP genotyping are more reliable when performed using appropriate template quantities. Figure 3 illustrates that signal strength for unknown samples should be similar to standards when performing SNP genotyping. Low template quantities make allele calls unreliable or impossible. Equal loading of different templates is an integral part of the experimental design in gene expression studies where samples are compared as treated versus untreated.

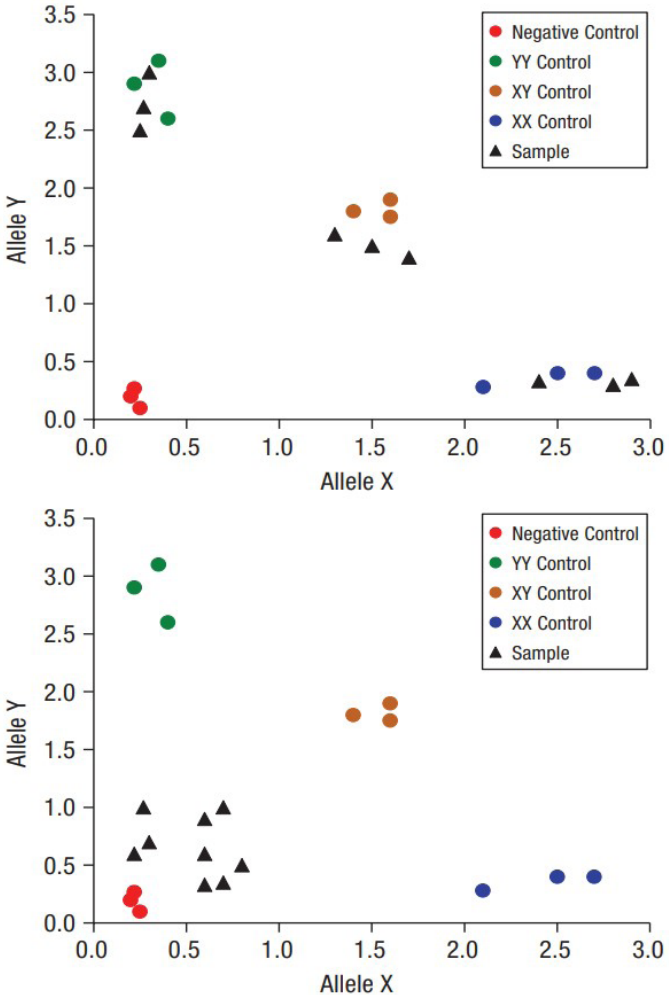

Figure 3. Example of SNP genotyping using appropriate template quantities (top) and sub-optimal template quantities (bottom). Note with appropriate template quantities, allele calls for unknown samples are clear, while low template quantities make allele calls unreliable or impossible.

A more common instance where exact quantification is critical is the validation of new reference genes. Validation of 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 a probe, if relevant) for the candidate gene, and the Cq is measured for each sample. Statistical analysis of Cq cycle numbers is then performed to test for a difference between 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.

Why is Purity Important?

Residual chemical contamination from extraction procedures can drastically influence downstream analysis; however, many contaminants are detectable using NanoDrop spectrophotometers.[3,4] Figure 4 shows a purified DNA sample, along with the same sample contaminated with protein and phenol. Upon initial examination, both contaminated samples appear to yield a spectra similar to "pure" nucleic acids, and in fact, the A260/A280 ratios seen for phenol contamination are often normal.[5]

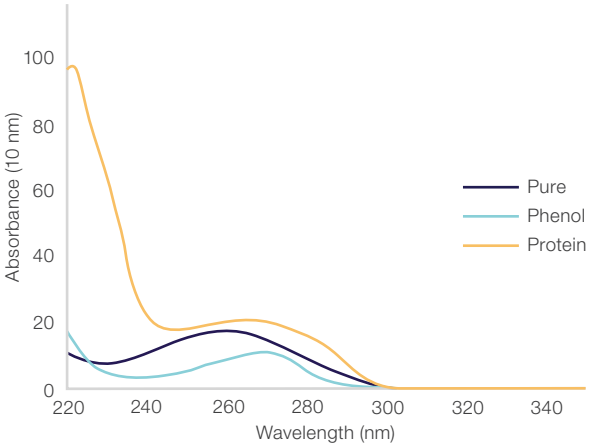

Figure 4. Spectra of purified dsDNA without contamination (dark blue), and of the same DNA sample contaminated with phenol (light blue) and protein (yellow).

The Thermo Scientific™ Acclaro™ Sample Intelligence Technology built into the NanoDrop One/One^{C} and NanoDrop Eight software circumvents these concerns. By utilizing a chemometric approach, the Acclaro Contaminant Identification feature analyzes the chemical components present in a sample and identifies common sample contaminants (Figure 5). This feature eliminates the guesswork required for analyzing and troubleshooting purity ratios and spectra.

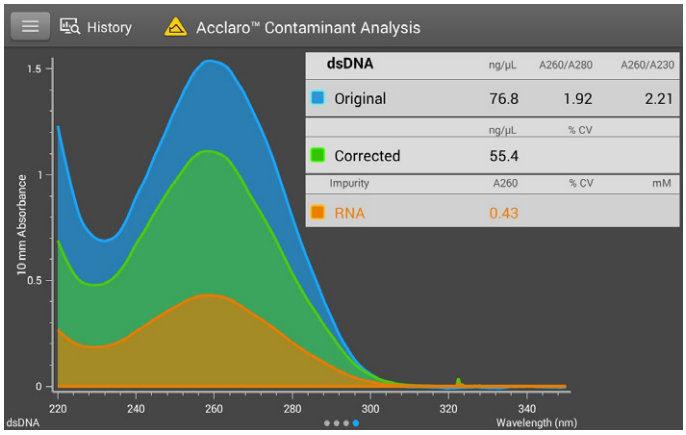

Figure 5. The Acclaro contaminant analysis from the NanoDrop One instrument identifying RNA as an impurity in a dsDNA sample. The original, uncorrected spectrum is in blue, corrected dsDNA spectrum in green, and the RNA impurity in orange. The Acclaro technology also provides a corrected dsDNA concentration in the table on the right.

For other UV-Vis spectrophotometers that do not utilize the Acclaro technology, such as the NanoDrop Lite Plus instrument, purity ratios may assist in identifying a contaminant. Many contaminants which cause failures in downstream applications absorb at around 230 nm or less. In addition to examining the A260/A280 ratio and the general shape of the spectra, we recommend the following:

- Check the A260/A230 ratio – a low 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, such as phenol, however many contaminants share one similar characteristic: absorbance at 230 nm or less. Absorbance at 230 nm may indicate the need to modify extraction procedures. High absorbance at 230 nm, or a poor A260/A230 ratio, may be caused by the following:

- Carbohydrate carryover (common in plant extraction)
- Residual phenol from nucleic acid extraction
- Residual guanidine (often used in column-based kits)
- Glycogen used for precipitation

Conclusion

The MIQE guidelines stipulate that a measurement of nucleic acid quantity is essential, while an assessment of purity is desirable. The measurement of both quantity and purity is beneficial not only to design better experiments but also to improve reporting. The use of a NanoDrop spectrophotometer to examine nucleic acids can result in significant savings in time and money by preventing the need to re-run downstream reactions. Extraction clean-up steps are small delays compared to repeating an entire experiment like qPCR.

References

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