Application note

Using NanoDrop One/One^{C} to determine phenol and protein contaminants in nucleic acids for RT-qPCR quality control

Abstract

For an accurate, reliable, and successful RT-qPCR experiment, several factors must be considered. To start, template RNA must be added to the reactions in accurate concentrations and be free of contaminants. The Thermo Scientific™ Acclaro™ Sample Intelligence technology integrated in the Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer software determines contaminants such as proteins and residual extraction reagents including phenol. In this study, the effect on RT-qPCR results from spiking RNA samples with phenol, TRIzol, bovine serum albumin (BSA), and hemoglobin was investigated, using the BRCA1 TaqMan assay. The results indicate that spiking RNA with phenol, TRIzol, and proteins increases the quantification cycle (C_q) and causes inaccuracies in the absorbance concentration results. The advantage of Acclaro technology to determine contaminants prior to RT-qPCR and reporting an accurate, corrected concentration will save significant time and resources by preventing failed reactions.

Introduction

Reverse transcription quantitative PCR (RT-qPCR) is an established molecular biology technique used for various applications including gene expression assays. According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, it is crucial to maintain quality control of RNA samples, otherwise variability in PCR results will be demonstrated. Sample quality control includes maintaining RNA preparations that are free of DNA and extraction reagents. The MIQE guidelines suggest measuring the A260/A280 purity ratio to determine purity of RNA samples.[1] The A260/A280 purity ratio for pure RNA samples is widely considered to be about 2.0 and contaminating analytes can influence this value. While a purity ratio is an acceptable method of determining purity, the ratio does not provide insight into the contaminant identity because most extraction material, such as phenol, absorb in the same region of the spectrum as nucleic acids.

With the large number of nucleic acid extraction kits, reagents, and protocols available, it is difficult to determine which kit or protocol delivers the purest product. Residual extraction materials may carry over to the extracted nucleic acid product and further protocol optimization would be required before continuing with the RT-qPCR reaction. Contaminating materials will influence the quantification cycle (C_q), thus purity must be properly determined prior to loading nucleic acid to the reaction mix.[2] Protein and phenol contamination cause inhibition of the DNA polymerase during PCR amplification and phenol also denatures the reverse transcriptase enzyme, both instances leading to false negative results.[3,4] In addition to ensuring the sample is pure, an accurate concentration must be determined to confirm precise amounts of template RNA are added to the reaction wells. The MIQE guidelines suggest measuring concentration prior to loading the sample in a PCR plate to ensure homogeneity between replicates.[1] Additionally, some contaminating material, such as those found in common extraction kits, will overestimate the nucleic acid concentration. If sample concentration is inaccurate, the results will vary between wells or if the sample is too dilute, this may reduce primer binding capacity and influence the RT-qPCR C_q results.[5]

NanoDrop One/One^{C} Microvolume UV-Vis Spectrophotometers

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Advantage of NanoDrop One Spectrophotometer in RT-qPCR workflow

To help ensure nucleic acid samples are free of contaminants that affect RT-qPCR results, the Thermo Scientific Acclaro Sample Intelligence technology identifies common contaminants in nucleic acid preparations and reports a corrected sample concentration (Figure 1). The Acclaro technology is integrated into the NanoDrop One/One^{C} Spectrophotometer local software and PC software. A well-known nucleic acid extraction procedure, the phenol-chloroform procedure, produces pure and undegraded RNA but has the potential for reagents to carry over in the extracted RNA product.[6] Another common nucleic acid extraction reagent, TRIzol, contains phenol and guanidine isothiocyanate, which are both identified in RNA preparations with the Acclaro technology.[7] The TRIzol based method of extraction separates RNA to the aqueous phase, DNA to the interphase, and protein to the organic phase.[7] The phenol-chloroform and TRIzol extraction methods have significant potential for phase carry-over if the researcher is not vigilant in their pipetting, resulting in a contaminated nucleic acid product.

In addition to the Acclaro technology features, the NanoDrop One/One^{C} Spectrophotometer utilizes microvolume sample measurements, conserving the extracted RNA product for downstream RT-qPCR optimization, if needed. In less than 8 seconds, the NanoDrop One/One^{C} instrument reports the A260/A280 and A260/A230 purity ratios along with the sample concentration without the need for dilutions, providing helpful details prior to the RT-qPCR reaction. Figure 2 outlines the contaminants that are identified by the Acclaro technology for the dsDNA and RNA applications. The software will report the original concentration without any Acclaro algorithm correction applied, a corrected sample concentration, and the contaminant's absorbance contribution to the analytical wavelength.

dsDNA	RNA
Protein	Protein
Phenol	Phenol
Guanidine HCl	Guanidine isothiocyanate
RNA	DNA

Figure 2: Contaminants identified by the NanoDrop One/One^{C} instrument's Acclaro technology for the RNA and dsDNA applications.

Materials and method

- Total RNA from human lymphocytes (isolated by BioChain, R1254148-1) was prepared by dialyzing and diluting in Tris-EDTA (TE) buffer (Fisher BioReagents, pH 8.0, BP2473500). 25 ng/µL RNA samples were spiked separately with phenol (Fisher BioReagents, BP1750B-65), bovine serum albumin (Sigma Aldrich, A7284-50ML), hemoglobin (MP Biomedicals, 02100714-CF), and TRIzol (QIAzol by Qiagen, 79306).
- Spiked RNA samples were then measured on the NanoDrop One/One^{C} instrument to determine the corrected and original RNA concentrations reported by the Acclaro technology. The results are shown in Figure 3. The original, uncorrected concentration was subsequently diluted to 25 ng/µL prior to loading on the qPCR plate to mimic that of a spectrophotometer without the Acclaro technology.
- BRCA1 TaqMan® Gene Expression Assay (Applied Biosystems, Hs01556193_m1) served as the target. BRCA1 was amplified for each sample and standard using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems, 4444432). RT-qPCR was conducted on the Thermo Scientific™ QuantStudio™ 6 Pro Real-Time PCR System (Applied Biosystems, A43159).

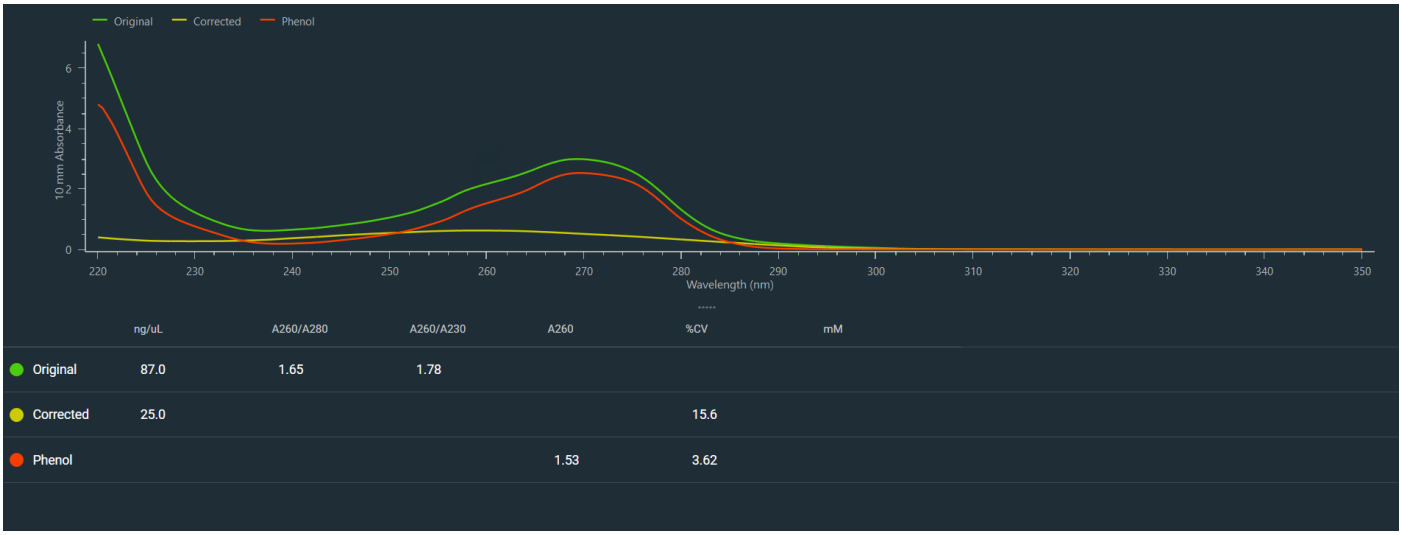

Figure 1: RNA spectra with phenol contamination, identified by Acclaro technology. The NanoDrop One/One^{C} software reports the corrected RNA concentration and spectrum in yellow.

Sample	Acclaro flag	Contaminant concentration	NanoDrop original concentration (ng/µL)	NanoDrop corrected concentration (ng/µL)
Control	N/A	N/A	99.8	N/A
BSA*	Yes	50 mg/mL	291.7	116.0
Phenol**	Yes	1000 ppm	87.0	25.0
Hemoglobin*	Yes	50 mg/mL	420.4	N/A***
TRIzol**	Yes	50A	277.6	101.1

* RNA concentration target in spiked sample was 100 ng/µL.
** RNA concentration target in spiked samples was 25 ng/µL.
*** Corrected concentration not reported by Acclaro Software due to high extinction coefficient of hemoglobin and low concentration of hemoglobin stock solutions.

Figure 3: The concentrations of RNA samples spiked with contaminants were measured on the NanoDrop One/One^C Spectrophotometer to trigger Acclaro Contaminant ID analysis using the RNA application.

Phenol and protein spike results

After the RNA samples were spiked with phenol, TRIzol, BSA, and hemoglobin separately, the samples were measured with the RNA application on the NanoDrop One/One^C Spectrophotometer. In Figure 3, the original and corrected RNA concentrations were provided by the Acclaro technology. For the "corrected" samples, the corrected concentration reported by the Acclaro Software was used in calculations to dilute to the 25 ng/µL target concentration for loading onto the PCR plate. For the "original" samples, the original, uncorrected concentration reported by the Acclaro Software was used to dilute samples to the 25 ng/µL target concentration for loading onto the PCR plate.

Upon completion of RT-qPCR, the standard curve resulted in an R^2 of 0.998 with 97.5% efficiency. The QuantStudio Design and Analysis Software calculated the mean C_q and the values are expressed in Figures 4 and 5.

For the phenol and TRIzol experiment, the control had a C_q of 24. The phenol spiked sample had a C_q of 28.5 for the original concentration and 26.6 for the corrected concentration. For TRIzol, the C_q of the original concentration was 25.3 and 23.8 for the corrected concentration. For the protein experiments, the control had a C_q of 24. The BSA spiked sample had a C_q of 31.4 for original concentration and 27.4 for the corrected concentration. For hemoglobin, the C_q for the original concentration was 28.8 and 27.9 when the corrected concentration was estimated to be 100 ng/µL.

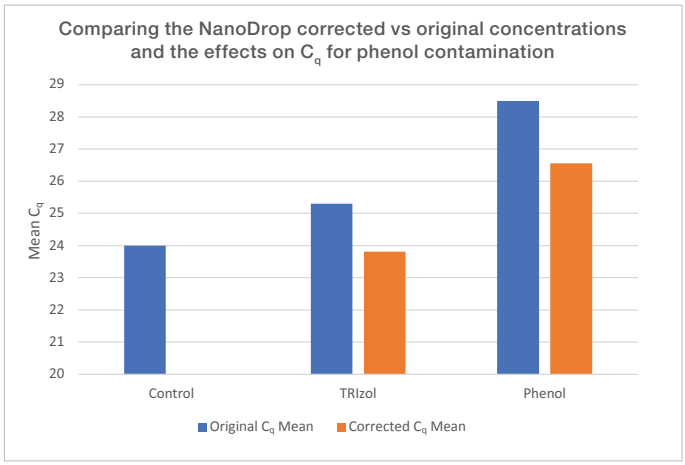

Figure 4: The effect on C_q mean results for phenol-spiked RNA samples using the corrected and original, uncorrected RNA concentration provided by the Acclaro Software. The blue bar represents the C_q mean results using the original concentration. The orange bar represents the C_q mean results using the corrected concentration provided by Acclaro analysis.

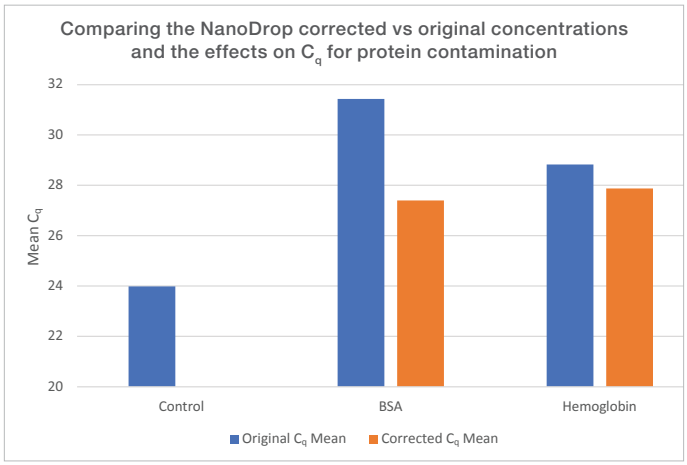

Figure 5: The effect on C_q mean results for protein-spiked RNA samples using the corrected and original, uncorrected RNA concentration provided by the Acclaro Software. The blue bar represents the C_q mean results using the original concentration. The orange bar represents the C_q mean results using the corrected concentration provided by Acclaro analysis.

Conclusion

An accurate RT-qPCR experiment requires the starting template RNA to be a specific concentration and be free of contaminants. Quality control must be implemented prior to downstream application because contaminating analytes can alter the sample concentration, thus influencing the C_q of the PCR assay. Historically, the A260/A280 purity ratio served as RT-qPCR quality control and while this is acceptable for determining nucleic acid purity, information regarding a contaminant is not provided. The Acclaro technology built into the NanoDrop One/One^C instrument determines the contaminating analyte and reports a corrected sample concentration. The indication of the sample contaminant provides helpful details to the experimenter and subsequent protocol optimization can be applied prior to RT-qPCR. The results outlined above accentuate the high sensitivity of RT-qPCR to protein or phenol contamination in RNA samples. With increasing amounts of phenol or protein, the RNA concentration was overestimated and the C_q value was also inflated as the amount of template was reduced with dilutions. Since phenol and protein are polymerase inhibitors, it is not expected that the C_q will be similar to the control when using the corrected concentration, but the Acclaro Software reports valuable contamination data for the user prior to RT-qPCR. Implementing the NanoDrop One/One^C Spectrophotometer into the RT-qPCR quality control workflow will save time and valuable resources for high-throughput applications.

References:

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