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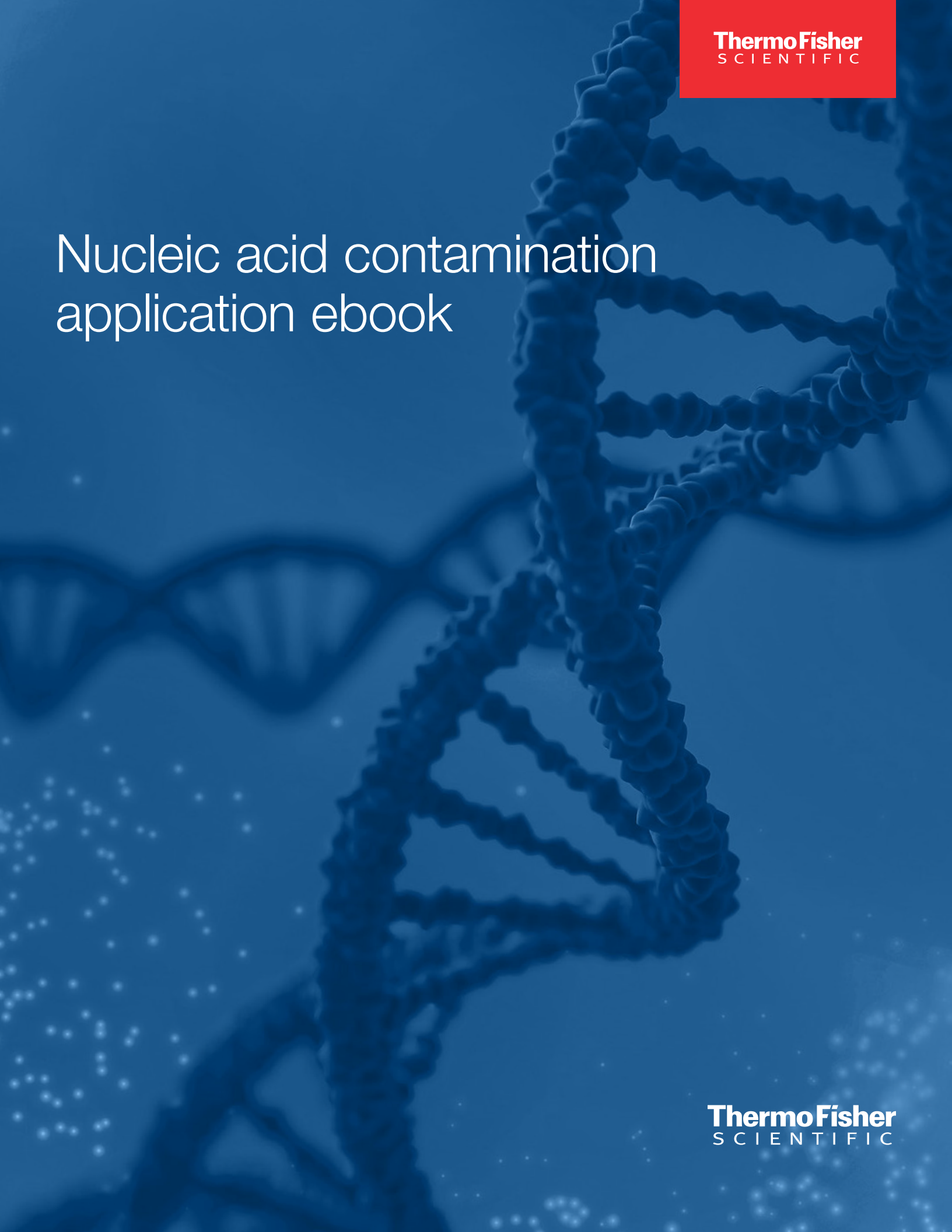
Nucleic acid contamination application ebook



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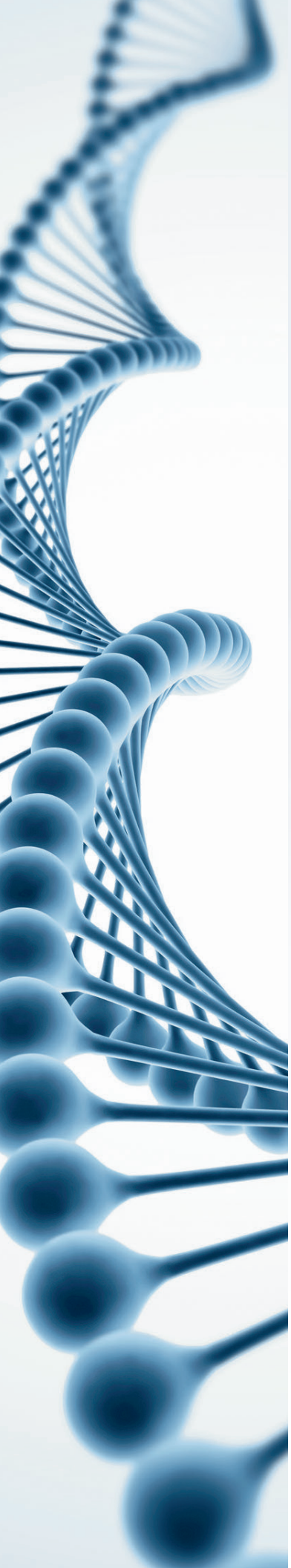

In this post-genomic era, the quantification of nucleic acids remains an essential technique used in modern laboratories today. Most cutting-edge biological and biomedical research uses experimental techniques and workflows involving nucleic acid samples. Experimental techniques such as PCR, qPCR, next-generation sequencing, and cloning require researchers to determine sample purity as well as other important variables affecting nucleic acid samples before using them in downstream experiments. Sample concentration, purity, and quality are the main sample variables that can be obtained by various instruments available in the life science market.

The quantification of nucleic acids has traditionally been performed by absorbance measurements at 260 nm. One thing to consider when evaluating samples using absorbance is that contaminants from the nucleic acid extraction process absorb in various regions of the UV spectrum. The presence of any of these contaminants in a nucleic acid sample can directly affect the accuracy of the quantification result.

This ebook presents several application notes about a chemometric approach to analyze the chemical components present in a sample. This type of analysis has been used previously in Fourier transform infrared spectroscopy (FTIR) and near-infrared (NIR) systems. The software algorithms in this ebook's application notes rely on a reference library of spectra. The algorithms are then applied to the sample spectrum, and the software can make predictions about a sample's contaminants by using chemometric mathematical principles. With these algorithms, scientists can accurately determine sample concentration even in the presence of contaminants absorbing light in the UV spectrum.

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TECHNICAL NOTE

No. TN52810

Nucleic acid measurements at 260 nm

NanoDrop One performance data

Authors

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Thermo Fisher Scientific, Wilmington, DE

Abstract

Life scientists can quantify nucleic acid samples on the Thermo Scientific™ NanoDrop™ One and One^{C} Microvolume UV-Vis Spectrophotometers using the preprogrammed applications for dsDNA, ssDNA, RNA, oligo DNA and oligo RNA (Figure 1). This technical note illustrates NanoDrop One Spectrophotometer performance across the instrument's dynamic range using dsDNA.

Figure 1: NanoDrop One software, nucleic acids Home screen

Introduction

The NanoDrop One Spectrophotometer is capable of accurately measuring samples ranging in concentration from 2 to 27,500 ng/µL dsDNA (0.04–550A) using as little as 1 to 2 µL of sample. The patented* sample-retention system and auto-ranging pathlength technology of the NanoDrop One/One^{C} instruments allow users to measure samples spanning a wide concentration range, thus eliminating the need for dilutions or consumables.

With the NanoDrop One instrument, no prior knowledge of sample concentration is needed to ensure measurement accuracy because sample concentration is essentially always within the dynamic range of the instrument. The NanoDrop One instrument was evaluated for accuracy across this dynamic range by comparing nucleic acid sample results to those obtained using the cuvette-based, benchtop Thermo Scientific™ Evolution™ 300 UV-Vis Spectrophotometer. The data presented here demonstrates that the NanoDrop One/One^{C} instruments accurately measure dsDNA samples as dilute as 3 ng/µL and as concentrated as 28,000 ng/µL (and up to 18,150 ng/µL ssDNA and 22,000 ng/µL RNA).

NOTE: the NanoDrop One^{C} instrument contains both a pedestal and a cuvette measuring position. The data presented here was collected on a NanoDrop One instrument and reflects the accuracy of pedestal measurements on both NanoDrop One and NanoDrop One^{C} instruments.

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Method

A series of solutions, ranging from 3 to 28,000 ng/µL, were prepared by diluting dsDNA sodium salt from salmon testes (Sigma Aldrich®, #D1626) in HPLC grade ddH_2O (Acros Organics™, #268300040). The initial concentration of the prepared dsDNA stock was validated spectrophotometrically at 260 nm using the Evolution 300 Spectrophotometer as a reference. Samples that fell outside of the absorbance detection limits of the reference spectrophotometer were diluted to fall within the working range of the reference instrument. The concentration values obtained for the diluted samples on the Evolution 300 instrument were used to manually calculate the original concentration of the dsDNA samples; the appropriate factors were applied to account for dilutions and pathlength differences as needed. The absorbance of each solution was then measured on a NanoDrop One instrument using the dsDNA application program. Sample sizes used to measure concentrations for each instrument were; 1.) 1 µL for the NanoDrop One instrument, 2.) 3 mL in a 10 mm quartz cuvette for the Evolution 300 instrument, and 3.) 1 µL in the Hellma® Analytics TrayCell cuvette-like device for the Evolution 300 instrument.

To calculate the concentration of each sample, the NanoDrop One software uses the measured absorbance at 260 nm, the mass extinction coefficient for dsDNA (50 ng/µL cm^{-1}) and Beer's Law. The average concentration values acquired from both the NanoDrop One and the Evolution 300 (Table 1) Spectrophotometers were plotted (Figures 2 and 3) and analyzed. These results demonstrate the high degree of measurement agreement between the two spectrophotometers.

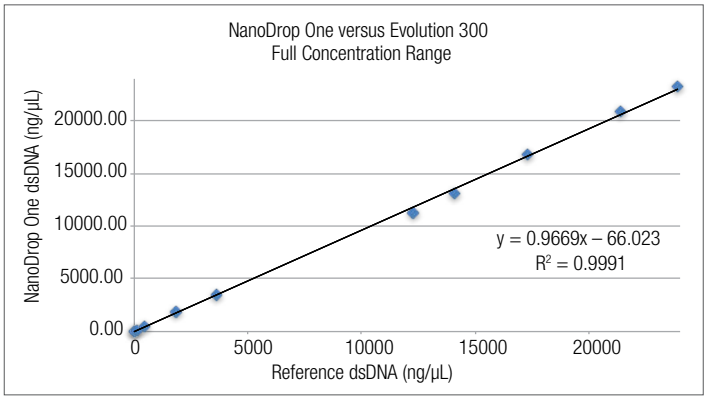

Figure 2: A linearity comparison between the NanoDrop One versus Evolution 300 Spectrophotometers across the entire instrument concentration range was plotted. The regression line demonstrates that the NanoDrop One dsDNA concentration results were well aligned with the values obtained on the Evolution 300 Spectrophotometer.

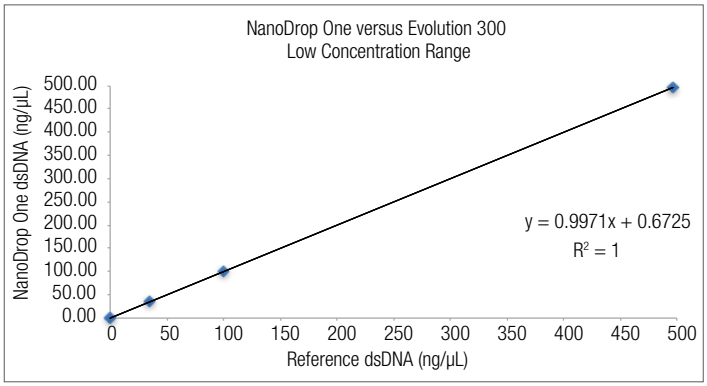

Figure 3: A closer look at the linearity comparison between the NanoDrop One and Evolution 300 Spectrophotometers at the low concentration range was also plotted (from 35 to 495 ng/µL). The regression line shows a close correlation to the Evolution 300 results and superb linearity at the lower end of the detection range.

Evolution 300 Spectrophotometer		NanoDrop One Spectrophotometer		
dsDNA [Conc] (ng/µL)	% CV	dsDNA [Conc] (ng/µL)	Standard Deviation	% CV
3.4	0.43	3.9	0.5	N/A
34.3	0.29	35.8	0.3	N/A
100.1	0.29	100.1	0.6	0.1
496.2	0.23	495.5	0.6	0.0
1844.5	0.33	1815.8	7.3	0.0
3606.4	0.25	3389.0	9.5	0.0
12259.8	0.10	11170.1	70.6	0.1
14095.8	6.81	13108.7	57.8	0.0
17289.4	1.97	16789.2	115.1	0.1
21338.6	3.74	20896.0	122.6	0.1
23887.3	3.24	23217.9	199.0	0.1

Table 1: Average concentration values from various preparations of dsDNA were measured both on a NanoDrop One and an Evolution 300 Spectrophotometer. Ten separate replicates of each solution were measured directly on the pedestal of the NanoDrop One instrument without further dilution. Solutions were diluted as necessary and measured in triplicate on the Evolution 300 Spectrophotometer fitted with a 10 mm quartz cuvette (Starna Cells, Inc., 1-Q-10) and a Hellma Analytics TrayCell micro-cell with a 0.2 mm cap.

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Published nucleic acid reproducibility specifications for the NanoDrop One Spectrophotometer are a standard deviation of ±2 ng/µL for sample concentrations between 2.0 and 100 ng/µL, and ±2% CV for samples >100 ng/µL measured on the pedestal. Table 2 presents reproducibility data from 10 replicates of dsDNA samples ranging in concentration from 3 to 28,000 ng/µL. The standard deviation data makes abundantly clear the superior measurement repeatability of the NanoDrop One Spectrophotometer. Measurement reproducibility exceeded specifications across the sample concentration range (Table 2).

Conclusion

The NanoDrop One Microvolume Spectrophotometer demonstrates excellent linearity across the complete dynamic range of the instrument (Figure 2) and superior linearity in the low concentration range (Figure 3). The calculated R^2 values in both cases show a close correlation between the concentrations obtained from the two spectrophotometers, the reference cuvette-based instrument and the NanoDrop One. The NanoDrop One instrument can produce this high level of reliability due to its patented*, pedestal sample-retention technology and automatic pathlength selection, so critical to managing microvolume measurements.

In addition to a high degree of accuracy across the concentration range tested, the NanoDrop One Spectrophotometer saves valuable time and money with its ease of operation and sample size requirement of only 1–2 µL. The NanoDrop One Spectrophotometer eliminates the need for sample dilutions and costly consumables such as quartz cuvettes. With pre-configured applications specifically designed for life science labs and an integrated high-resolution, touchscreen interface, the NanoDrop One instrument is fast and simple to use while being accurate and reliable.

**Patents US6628382 and US6809826*

Sample	1	2	3	4	5	6	7	8	9	10	11
Replicate 1	4.4	36.1	101.1	495.2	1799.5	3400.6	11005.2	13132.6	17004.4	21042.0	23155.9
Replicate 2	4.6	35.8	100.0	495.8	1820.5	3394.6	11121.2	13022.4	16873.8	21110.6	23159.4
Replicate 3	3.8	35.4	99.5	496.0	1821.6	3386.3	11159.4	12995.4	16817.8	20925.5	23332.2
Replicate 4	3.3	35.9	99.5	494.4	1818.6	3390.5	11169.7	13111.4	16750.4	20706.0	23276.3
Replicate 5	3.5	35.4	100.3	495.5	1812.8	3372.5	11170.5	13123.3	16765.5	20820.3	23519.3
Replicate 6	3.6	36.3	100.1	495.2	1824.9	3378.8	11126.0	13156.9	16781.7	20896.9	23337.1
Replicate 7	4.4	35.5	100.1	496.5	1814.0	3387.6	11200.5	13167.0	16829.4	20895.1	22991.7
Replicate 8	3.5	35.8	100.7	495.4	1810.1	3391.3	11251.0	13070.1	16557.3	20875.5	23439.8
Replicate 9	4.1	35.9	100.0	495.3	1817.6	3376.3	11217.0	13043.1	16703.5	20790.7	22890.0
Replicate 10	4.5	35.6	99.3	495.7	1810.6	3399.2	11231.8	13106.1	16796.8	21019.0	23077.6
Average	4.0	35.8	100.1	495.5	1815.0	3387.8	11165.2	13092.8	16788.0	20908.1	23217.9
Std Dev	0.5	0.3	0.6	0.6	7.3	9.5	70.6	57.8	115.1	122.6	199.0
% CV	N/A	N/A	0.06	0.01	0.04	0.03	0.06	0.04	0.07	0.06	0.09

Table 2: Measurement reproducibility was assessed on a NanoDrop One instrument using 10 separate aliquots of each concentration of dsDNA sodium salt from salmon testes. The average concentration, standard deviation, and % CV were calculated for each preparation. The measurement reproducibility across the dynamic range of the instrument meets and exceeds specification.

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TECHNICAL NOTE

No. TN52834

NanoDrop One sample contaminant identification

Frequently Asked Questions

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Frequently Asked Questions

The Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer is designed to help research scientists achieve success in downstream applications by accurately quantifying DNA, RNA and protein samples using only 1-2 µL. Built with novel Thermo Scientific™ Acclaro™ Sample Intelligence technology, the NanoDrop One instrument provides more information about sample quality by identifying common contaminants and delivering true sample concentrations. Here are answers to common questions about the Acclaro Contaminant Identification (ID) feature.

1. How does the Acclaro Contaminant ID feature work on the NanoDrop One Spectrophotometer?

The Acclaro Contaminant ID feature uses a chemometric approach to analyze the chemical components present in a sample. This type of analysis has been used previously in Fourier transform infrared spectroscopy (FTIR) and near-infrared systems (e.g., Thermo Scientific™ Nicolet Spectrometers). The Acclaro algorithms rely on a reference library of spectra. These algorithms are then applied to the sample spectrum, and the software can make predictions about a sample's contaminants by using chemometric mathematical principles.

2. In which NanoDrop One application is the Acclaro Contaminant ID feature implemented?

Contaminant ID is implemented in the dsDNA, RNA and Protein A280 Applications.

3. What contaminants can the Acclaro Contaminant ID technology detect?

The Acclaro reference libraries currently support detection of RNA, protein, phenol, and guanidine HCl in dsDNA samples. They also support detection of DNA, protein, phenol, and guanidine isothiocyanate in RNA samples. Finally, they allows detection of DNA in protein samples. In the future, additional contaminants will be added to the libraries.

4. Are the Acclaro Contaminant ID algorithms applied to all sample concentrations?

No, in the dsDNA and RNA applications the Acclaro Contaminant ID algorithms are applied to samples at different absorbance ranges based on the contaminant. Consult the table for detailed information. In the protein A280 application, the Acclaro Contaminant ID algorithms are applied to all samples regardless of concentration. The table to the right summarizes common contaminants detected by Acclaro sample intelligence technology.

NanoDrop One Application	Detection Wavelength	Detected Contaminants	Sample Concentration
dsDNA	260 nm	Protein Phenol Guanidine HCL	0.5A - 62.5A 25 - 3,125 ng/µL
		RNA	1.0A - 20A 50 - 1,000 ng/µL
RNA	260 nm	Protein Phenol Guanidine Isothiocyanate	0.5A - 62.5A 20 - 2,500 ng/µL
		DNA	1.0A - 20A 40 - 800 ng/µL
Protein	280 nm	DNA	All concentrations

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5. How do you read the Acclaro Contaminant ID results?

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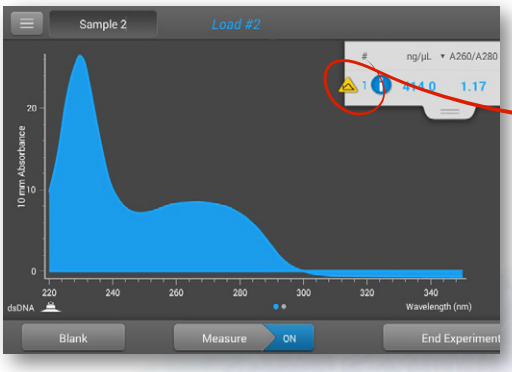
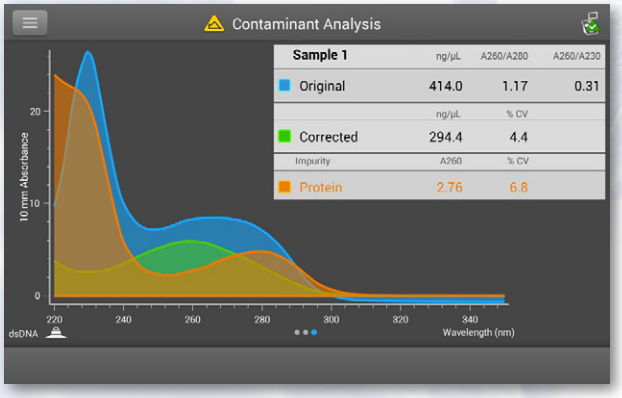


Acclaro Contaminant ID icon: The Acclaro algorithms have detected a contaminant in this dsDNA sample.

This Results screen shows that the Acclaro algorithms have detected contaminants in this DNA sample, as represented by the yellow triangle. To find out what contaminants the Acclaro software has identified and to obtain the corrected DNA concentration, simply tap the yellow icon to open the Contaminant Analysis screen shown below.



Original:
Concentration result without any Acclaro Contaminant correction applied. *Blue Spectrum*

Corrected:
Concentration result with Acclaro Contaminant correction applied. *Green Spectrum*

Impurity:
Contaminant that has been detected and how much 260 nm absorbance the contaminant contributed to 260 nm peak. *Orange Spectrum*

The Acclaro Contaminant Analysis screen presents important information about the sample. The original concentration and purity ratio results are shown on the top row. This is the calculated concentration result (Original) before contaminant corrections have been applied. The second row labeled “Corrected” shows two values. The first value is the corrected concentration of the analyte in the sample in ng/µL (the analyte in this example is dsDNA as indicated in the lower left corner of the screen). The Corrected result is the analyte concentration after contaminant correction has been applied. This value is the software-predicted concentration for the pure nucleic acid component in the sample. The second value is the %CV, which represents the confidence in the prediction. The lower the %CV number, the more confident we are in the corrected concentration predicted by the software. The third row identifies the impurity that is present (RNA, protein, phenol, guanidine HCl, or guanidine isothiocyanate) and reports how much absorbance at 260 nm is contributed by the contaminant. In this case, the %CV represents the confidence in the contaminant identity prediction.

6. How do we use Acclaro Contaminant ID results in workflows?

The Acclaro Contaminant ID feature gives customers two important pieces of information. First, the Contaminant ID feature identifies specific contaminants that are likely to be present in the sample. The identification of the potential contaminants in the sample can help scientists troubleshoot difficult extractions or purifications and make decisions regarding sample use in downstream experiments. Second, the Contaminant ID gives customers a corrected concentration. When setting up downstream reactions where DNA concentration is a critical parameter (e.g., PCR), the corrected concentration will help scientists ensure the success of downstream experiments.

7. What accuracy and sensitivity can I expect from the Acclaro Contaminant ID feature, and what factors affect the accuracy of the result?

Large sets of known mixtures of analytes “spiked” with contaminants were measured on the NanoDrop One instrument during the development of this feature. In general, the corrected nucleic acid result (the software-predicted concentration) was within 10% of the actual concentration. The sensitivity of Acclaro Contaminant ID depends on the contaminant’s molar absorptivity relative to the analyte of interest (nucleic acid or protein). Phenol has a very high molar absorptivity, therefore, it takes very little phenol to affect the spectrum and generate a corrected concentration for the analyte. Protein has a low molar absorptivity relative to dsDNA, therefore, it takes a large amount of protein to affect the spectrum.

Find out more at **thermofisher.com/nanodrop**

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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 to report 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 A_{260}/A_{280} purity ratio to determine purity of RNA samples.[1] The A_{260}/A_{280} 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]

Figure 1. 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 Acclaro Sample Intelligence technology identifies common contaminants in nucleic acid preparations and reports a corrected sample concentration (Figure 2). 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 A_{260}/A_{280} and A_{260}/A_{230} purity ratios along with the sample concentration without the need for dilutions, providing helpful details prior to the RT-qPCR reaction. Figure 3 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 3. 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). RNA samples (25 ng/µL) 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 4. 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).

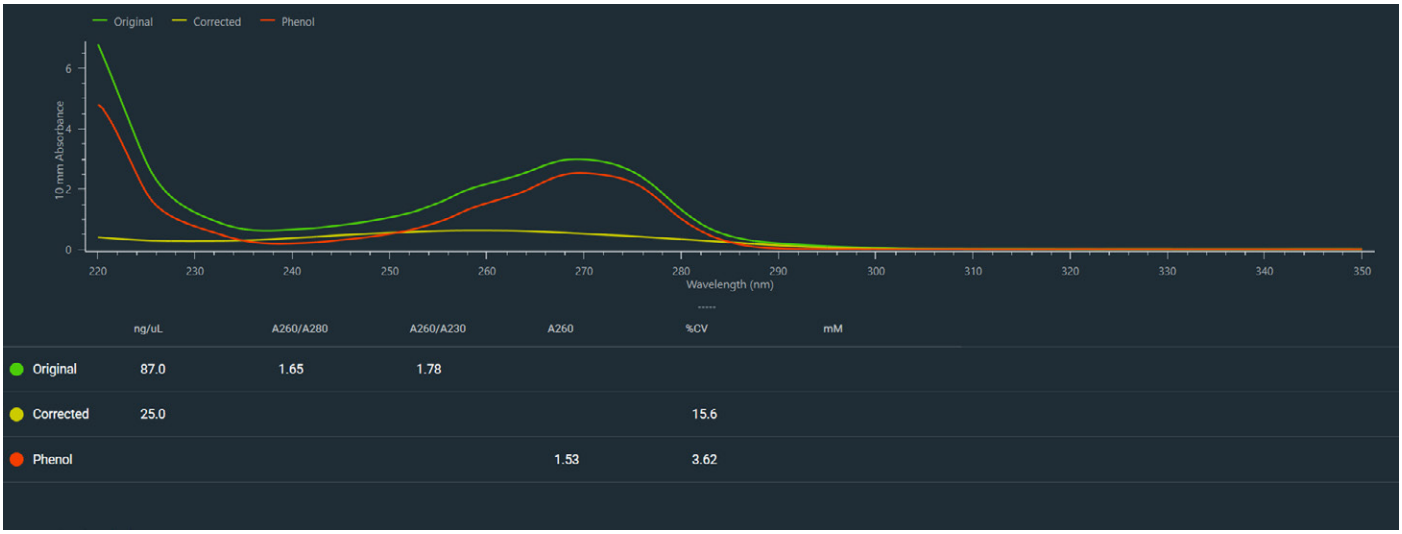

Figure 2. RNA spectra with phenol contamination, identified by Acclaro Technology. The NanoDrop One/One^{C} software reports the corrected RNA concentration and spectrum in yellow.

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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 4. The concentrations of RNA samples spiked with contaminants were measured on the NanoDrop One/One^C Spectrophotometer to trigger Acclaro Technology 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 4, 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 5 and 6.

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.

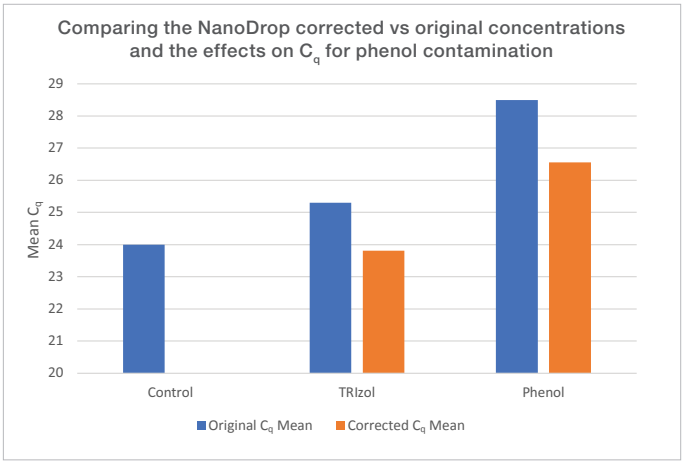

Figure 5. The effect on C_q mean results for phenol-spiked RNA samples using the corrected and original, uncorrected RNA concentration provided by the Acclaro Software. The blue bar represents the C_q mean results using the original concentration. The orange bar represents the C_q mean results using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

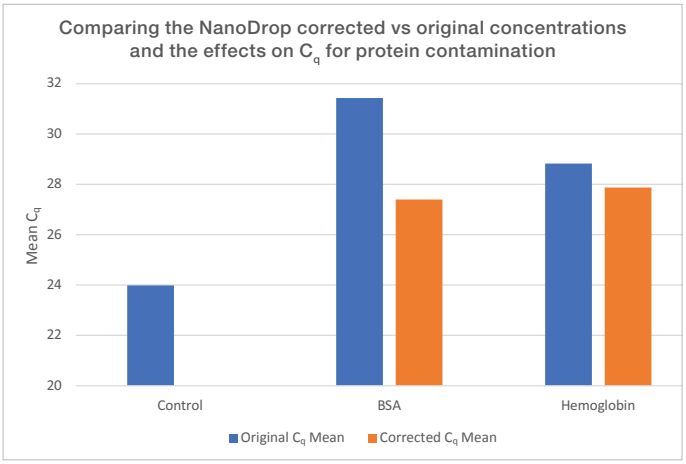

Figure 6. The effect on C_q mean results for protein-spiked RNA samples using the corrected and original, uncorrected RNA concentration provided by the Acclaro Software. The blue bar represents the C_q mean results using the original concentration. The orange bar represents the C_q mean results using the corrected concentration provided by Acclaro Technology's analysis.

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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 A_{260}/A_{280} 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.

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mRNA vaccine production: ensure quality control with the NanoDrop Spectrophotometers

Introduction

In the height of the SARS-CoV-2 pandemic, messenger RNA (mRNA) vaccines became increasingly popular in the fight to end the pandemic due to their safety, efficacy, and rapid production capabilities.[1] mRNA vaccines function by training cells to produce a foreign protein that is sufficient to yield an immune response. The subsequent immune response triggers the development of antibodies specific to the target protein, killing pathogenic viruses as they enter the body in the future.[2] In this note, the use of the Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer or the NanoDrop Eight Microvolume UV-Vis Spectrophotometer in the mRNA vaccine manufacturing protocols as a quality control checkpoint was investigated.

Sequencing

The early production of mRNA vaccines begins with an extraction of nucleic acids and preparation for sequencing. To successfully sequence nucleic acids, it is crucial that the starting material is of a certain quality and quantity.[3] The Genomics Core Facility at the Pennsylvania State University requests total RNA for sequencing that is free of contaminants, since any foreign material can cause "variance…in the transcript profile." With the large number of nucleic acid extraction kits and protocols available, the concentration yield and purity can vary with each kit or protocol. Implementing the NanoDrop One/One^{C} Spectrophotometer or the NanoDrop Eight Spectrophotometer after the nucleic acid extraction step can serve as a quality control checkpoint prior to sequencing.

The Thermo Scientific™ Acclaro™ Sample Intelligence Technology, which is built into the NanoDrop One/One^{C} instrument and the NanoDrop Eight Spectrophotometer software, serves as an important factor in quality control. Historically, nucleic acid purity ratios, A_{260}/A_{280} and A_{260}/A_{230}, served as the method for quantifying purity. However, for traditional UV-Vis spectroscopic measurements, any material that absorbs at the analysis wavelength will contribute to the overall absorbance and concentration calculations. For example, contaminating dsDNA in an RNA preparation will co-absorb as both nucleic acids absorb at 260 nm, thus overestimating the concentration. To solve this drawback, the Acclaro Technology utilizes chemometric algorithms to distinguish RNA and dsDNA as well as common extraction reagent materials such as phenol and chaotropic agents. The Acclaro Technology provides an advantage to users in the sequencing workflow because contaminated RNA will interfere with the library production.[3] Once the genome has been sequenced successfully, a vaccine target is determined and plasmid production proceeds.

Vaccine production workflow

Figure 1. mRNA vaccine workflow displaying the integration of the NanoDrop One/One^{C} Spectrophotometer or the NanoDrop Eight Spectrophotometer as quality control checkpoints.

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Plasmid production

After completion of the sequencing process, the recombinant plasmid DNA is constructed by inserting the target gene in the plasmid via restriction endonucleases and a DNA ligase. The recombinant plasmid is transformed into *Escherichia coli* (*E. coli*), where the bacterial cells are then picked based on antibiotic resistance selection or colony color screening.[4] After the plasmid-containing colonies are selected and grown in a medium, the OD 600 of the culture is measured on a NanoDrop Spectrophotometer to determine whether the density is appropriate for plasmid purification, typically 2.0–4.0 OD at 600 nm depending on the purification kit.

The plasmid is next purified from the cell culture with a kit to remove genomic DNA or other contaminants. Contaminating salts or proteins will affect downstream success, causing experiments to fail and forcing the user to troubleshoot, which is time consuming and costly. To mitigate downstream troubleshooting of failed experiments, the purified plasmid is measured on the NanoDrop One/One^C Spectrophotometer or the NanoDrop Eight Spectrophotometer, where the Acclaro Technology algorithms can identify residual contaminants post-purification. The additional quality control measures for plasmid production can be easily implemented with the NanoDrop Spectrophotometers to provide full spectral data in fifteen seconds or less, depending on the instrument model.

mRNA synthesis: *In vitro* transcription

When the recombinant plasmid is determined to be pure from measuring on a NanoDrop Spectrophotometer, the plasmid is linearized and the polymerase begins transcription ahead of the gene of interest. The polymerase synthesizes mRNA from the DNA template and the mRNA is capped to ensure efficient translation to protein.[5] When the mRNA synthesis is complete, the DNA is degraded with DNase, and the mRNA undergoes a final purification to remove contaminating transcription materials.[1] As a final quality control checkpoint with the NanoDrop Spectrophotometers, the purified mRNA is checked for purity before filling the vaccine vials with the mRNA solution.

The Acclaro Technology available on the NanoDrop One/One^C Spectrophotometer and the NanoDrop Eight Spectrophotometer provides an enhanced quality control checkpoint post-synthesis. It is crucial to determine purity of the final mRNA as contamination can reduce the vaccine potency via protein translation inhibition.[1] The contaminant identification and helpful onboard troubleshooting features of the Acclaro Technology eliminate the guesswork of determining purity from the A_{260}/A_{280} and A_{260}/A_{230} ratios and ensures a pure final mRNA product.

Conclusion

The NanoDrop One/One^C Spectrophotometer and the NanoDrop Eight Spectrophotometer enhance the quality control mechanisms of mRNA vaccine production from start to finish. The automated pathlength feature of all NanoDrop Spectrophotometers allows the user to measure an increased concentration range of 1–2 µL without the need for diluting samples. Along with the microvolume measurements, the NanoDrop instruments provide full spectral data in 15 seconds or less, depending on the instrument model.

With software available for the NanoDrop One/One^C Spectrophotometer and the NanoDrop Eight Spectrophotometer that allows users to comply with 21 CFR Part 11, the instruments are ready to be implemented into the mRNA vaccine workflow at a GMP laboratory. The addition of the Acclaro Technology built into the software allows the user to identify contaminants and provides recommended troubleshooting steps, saving overall cost and time from repeating failed experiments. With the increased interest in mRNA vaccines, the NanoDrop Spectrophotometers provide a quick and simple method for improving quality control.

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APPLICATION NOTE

No. AN53374

Detection of RNA contamination in Mammalian DNA preparations

Using advanced UV-Vis algorithms to improve specificity

Introduction

Quantification of nucleic acids has traditionally been performed by determining the UV absorbance at 3 analytical wavelengths: 230 nm, 260 nm, and 280 nm. The information derived from these absorbance measurements allows scientists to measure nucleic acid concentration and to have an indication of sample purity. A disadvantage of this approach is the lack of specificity. Thus, any contaminant that absorbs at these wavelengths will lead to inaccuracies in a nucleic acid concentration result.

The Thermo Scientific™ NanoDrop™ One/One^{C} Spectrophotomer features Thermo Scientific™ Acclaro™ Intelligence Technology Software which uses full-spectrum data and advanced algorithms to identify common nucleic acid contaminants and provide corrected nucleic acid concentrations. In this technical note, we show how a newly introduced feature in Acclaro software allows users to detect and correct for RNA contamination in DNA preparations or DNA contamination in RNA preparations.

Materials and methods

DNA and RNA were prepared from HeLa cells, treated with RNase or DNase to remove contaminant nucleic acid, and dialyzed in TE buffer, pH 8.0. Initial stock concentrations were determined on the NanoDrop One spectrophotometer against a TE blank. Various mixtures of DNA and RNA were made (as shown in Figure 1), and triplicates of each sample were read on the instrument. A fresh 2.0 µL aliquot of the appropriate mixture was used for each replicate. The following DNA concentration results were calculated:

- Theoretical DNA concentration: baseline corrected A260*50 ng/µL *(%DNA/100).
- Uncorrected DNA concentration: baseline corrected A260 *50 ng/µL.
- Acclaro-corrected DNA concentration: Acclaro-corrected A260 *50 ng/µL.

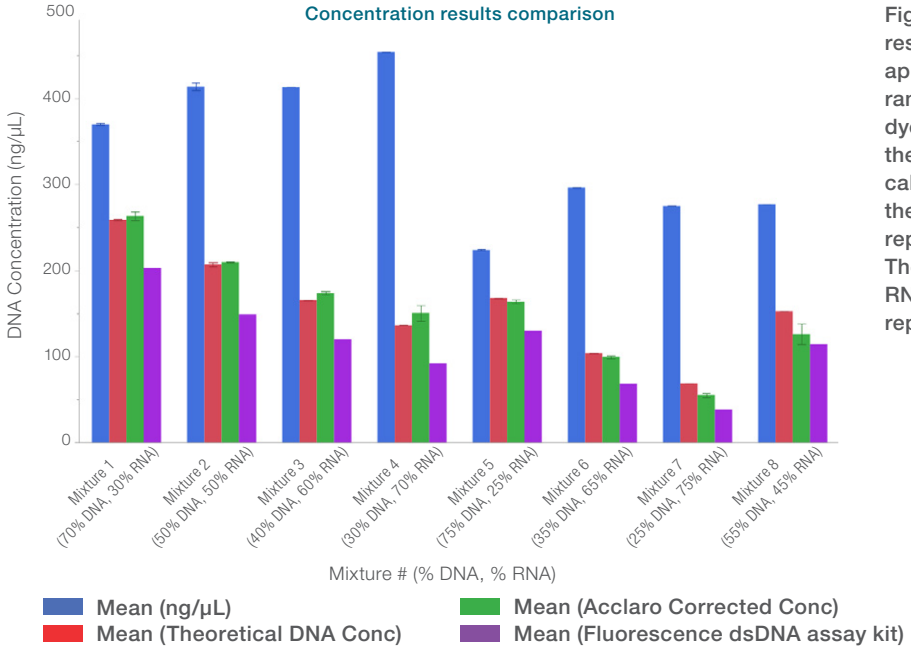

Figure 1: The bar graph compares the concentration results of various mixtures of DNA and RNA using different approaches. The purple bars represent data using a broad range dsDNA assay kit. This assay uses a fluorescence dye, that specifically binds DNA. The blue bars represent the data when a direct A260 absorbance is used to calculate the DNA concentration. The red bars represent the theoretical target DNA concentrations. The green bars represent the Acclaro-corrected DNA concentrations. The DNA and RNA preparations used for the DNA/RNA mixtures were isolated from HeLa cells. Error bars represent the standard deviation from the mean.

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The theoretical, uncorrected, and Acclaro-corrected DNA concentrations were compared to those obtained with a DNA-specific fluorescent assay. Fluorescence assays were performed using a broad range dsDNA assay kit and a fluorescence instrument. A standard curve was generated according to the assay kit's protocol. For this assay, standards were prepared by adding 10 µL of the appropriate DNA standard to 200 µL of working solution. Nucleic acid samples were then prepared by adding 2.0 µL of sample to 200 µL of working solution. DNA standards and samples were incubated at room temperature for 5 minutes and then read on the fluorescence instrument.

Mean DNA concentrations and standard deviations for each sample are shown in Figure 1.

NanoDrop One Spectrophotomer

Results

When calculating DNA concentration in the presence of contaminating RNA, the highest degree of inaccuracy was observed when uncorrected A260 absorbance was used to calculate the DNA concentration. Both macromolecules (i.e., RNA and DNA) absorb light at 260 nm; therefore, using absorbance at 260 nm to calculate the concentration of DNA can lead to an overestimation of the actual concentration of DNA in a sample.

The Acclaro-corrected results (green bars) demonstrate that greater accuracy is achieved in the determination of DNA concentration when using the Acclaro feature. The Acclaro algorithm uses the full spectral data in conjunction with multivariate mathematics to specifically determine the concentration of DNA in a DNA/RNA mixture. The DNA concentrations obtained after Acclaro correction were much closer to the those obtained by using a DNA-specific fluorescence dye.

Conclusion

RNA contamination in genomic DNA preparations is a common issue in molecular biology workflows. When measuring samples with traditional spectrometry, copurified RNA will artificially inflate the concentration of DNA. Although using specific DNA-binding dyes may provide a more accurate determination of DNA concentration than absorbance at 260 nm, contamination of a DNA sample with copurified RNA can have adverse effects in modern genomic workflows.

We demonstrated that by using full spectral data and multivariate mathematical algorithms, researchers can overcome the disadvantages of both type of assays mentioned. The Acclaro software that runs the Nanodrop One spectrophotometer allows the identification of RNA contamination in a DNA sample and provides a corrected concentration result. These two factors will allow molecular biologists to quickly troubleshoot difficult extractions and improve downstream results.

For more information about resolving nucleic acid contamination with NanoDrop One systems visit **thermofisher.com/NanoDropQuant**

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TECHNICAL NOTE

Acclaro phenol contaminant ID

Detection of phenol in nucleic acid samples using the NanoDrop One spectrophotometer

Authors: Sean Loughrey and Brian Matlock, Thermo Fisher Scientific, Wilmington, DE

Key Words
NanoDrop One, Acclaro Contaminant ID, Phenol Contamination, Purity Ratio, Chemometrics, UV-Vis, Spectrophotometer, Spectral Analysis, Sample Intelligence, DNA Quantification

Abstract
The new Thermo Scientific™ Acclaro™ Sample Intelligence technology built into the Thermo Scientific™ NanoDrop™ One microvolume UV-Vis spectrophotometers allows scientists to accurately quantify their nucleic acid samples in the presence of common contaminants carried over from nucleic acid extraction methods. In this study, we measured the concentration of DNA in the presence of various amounts of phenol contamination. We then compared the uncorrected versus the Acclaro-corrected concentration values. The results showed that the Acclaro-corrected DNA concentrations were within 10% of the concentration of the DNA-only control. The Acclaro-corrected results demonstrate the effectiveness of the software algorithm in identifying phenol contamination, properly correcting the DNA concentration values, and providing a more accurate concentration result than the A260 value alone. This information enables scientists to make informed decisions regarding sample use in downstream experiments and provides valuable information for troubleshooting problematic extractions.

Introduction
In this post-genomic era, the quantification of nucleic acids remains an essential technique used in modern laboratories today. Most cutting-edge biological and biomedical research uses experimental techniques and workflows involving nucleic acid samples. Experimental techniques such as PCR, qPCR, next-generation sequencing, and cloning require researchers to determine sample purity as well as other important variables affecting nucleic acid samples before using them in downstream experiments. Sample concentration, purity, and quality are the main sample variables that can be obtained by various instruments available in the life science market.

The quantification of nucleic acids has traditionally been performed by absorbance measurements at 260 nm. One thing to consider when evaluating samples using absorbance is that contaminants from the nucleic acid extraction process absorb in various regions of the UV spectrum. The presence of any of these contaminants in a nucleic acid sample can directly affect the accuracy of the quantification result.

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Reviewing purity ratios has been the primary method used to assess the presence of UV-absorbing contaminants. In general, researchers will verify that their purity ratios fall into an acceptable range (Table 1). However, relying on purity ratios alone does not provide a complete assessment of the potential contaminants in nucleic acid samples (Table 1). Purity ratios used in combination with full-spectral data greatly enhances the ability of researchers to determine not only the purity of a nucleic acid sample, but also an accurate concentration via A260 absorbance. Until now, analysis of a sample's spectrum has been a qualitative research endeavor, and the ability to identify specific contaminants from the spectrum has relied mostly on the researcher's analytical experience.

	260/280	260/230
DNA	1.8-2.0	1.8-2.2
RNA	2.0- 2.2	1.8-2.2
Pure Phenol	**1.6**	**2**

Table 1: Purity ratio ranges generally accepted for pure nucleic acid samples in TE buffer. Notice that the purity ratio for pure phenol does not deviate greatly from the purity ratios for pure nucleic acids.

Rigorous, quantitative research methods and data have driven the development of the Acclaro sample intelligence technology in the NanoDrop One spectrophotometer. The Acclaro technology provides a cutting-edge quantitative approach for contaminant identification by using chemometric methods to analyze the chemical components present in a sample. Ultimately, the goal for this new technology is to help scientists make informed decisions about whether to use their samples in downstream reactions and workflows that may be labor intensive, expensive or consume rare samples.

The NanoDrop One Acclaro Contaminant Identification (ID) feature allows for the detection of protein, phenol, and guanidine-salt contaminants in nucleic acid solutions. The NanoDrop One spectrophotometer alerts users to the presence of a contaminant in real time by displaying a yellow contaminant ID icon (Figure 1A) next to the sample number on the NanoDrop One touchscreen.

Tapping on the contaminant icon reveals the full contaminant analysis details provided by the Acclaro technology (Figure 1B). The screen displays the deconvolved spectra, identified contaminants, purity ratios, corrected DNA concentration, and the coefficient of variation, which represents the confidence in the Acclaro algorithm prediction. In this technical note, we present data obtained by the Acclaro Contaminant ID feature.

The data show how this rigorous, quantitative technology can accurately detect phenol contamination in nucleic acid samples and provide accurate DNA concentration values.

Phenol contamination in nucleic acid samples

The use of phenol to separate protein from nucleic acids has a long history, dating back to the 1950s. Although there have been various protocols and formulations developed over the years, the most popular technique was developed by Chomczynski and Sacchi in the mid-1980s. This technique used a mixture of guanidinium thiocyanate, phenol, and chloroform and allowed scientists to obtain highly pure, undegraded total RNA in a single step (Chomczynski and Sacchi, 1987, Chomczynski and Sacchi, 2006)[1-2]. This method served as the basis for the development of various RNA extraction kits including TRIzol™ kit (Thermo Fisher Scientific), TRI Reagent® kit (Molecular Research Center, Inc), QIAzol® kit (Qiagen), TriPure™ kit (Sigma-Aldrich), TRISure™ kit (Bioline), and RNAzol® kit (Molecular Research Center). These extraction kits use different formulations of phenol, guanidium thiocyanate, and chloroform to lyse cells and denature proteins including DNases and RNases. Traces of these reagents can be found in purified nucleic acid samples and may affect downstream workflows.

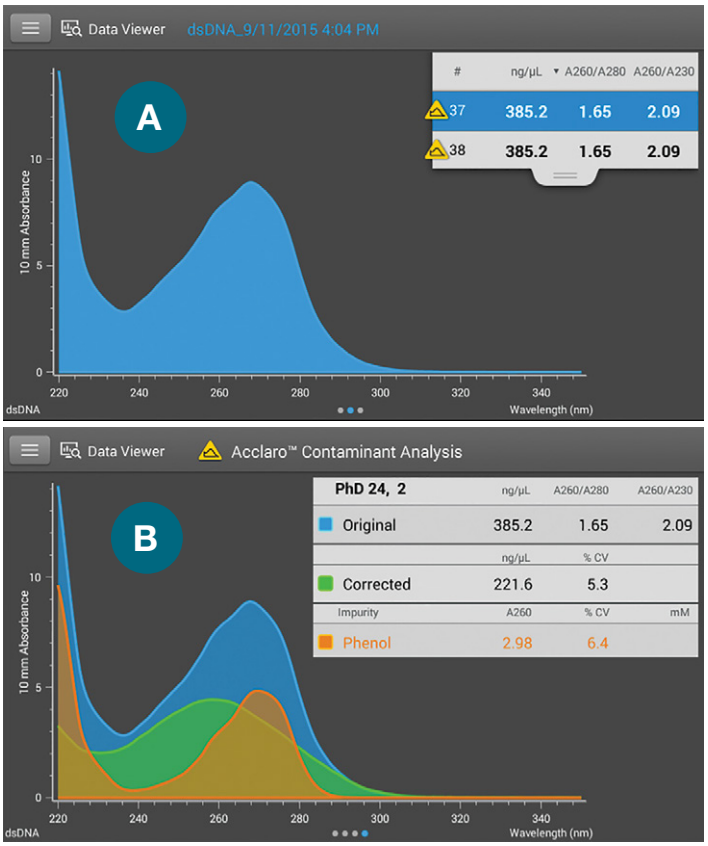

Figure 1: The Acclaro Contaminant ID feature identifies possible contaminants present in a sample. 1A) Measurement screen: the Acclaro Contaminant ID icon indicates that the Acclaro algorithms have detected a potential contaminant in a dsDNA sample. 1B) Contaminant analysis screen: comparison of original absorbance spectrum (DNA plus phenol, blue), the corrected spectrum (original minus contaminant, green) and the contaminant spectrum (orange). This screen also contains the original and corrected DNA concentration results.

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The extraction procedure will create an organic phase and an aqueous phase (Figure 2). The pH of the extraction solution will dictate which nucleic acid species is extracted into the aqueous phase. An acidic solution favors extraction of RNA into the aqueous phase, while an alkaline solution extracts both DNA and RNA into the aqueous phase. Partially denatured proteins congregate in the interphase between the two phases (Figure 2). Separation of nucleic acids from the denatured protein requires removal of the aqueous phase without disturbing the interphase (Figure 2). This can prove very difficult, especially for those who are new to the extraction technique. To avoid contamination of the purified nucleic acid with protein, phenol, or guanidium, removal of the aqueous phase requires experience and precise technique (Oswald, 2016, Plank, 2010, Jankovic 2016)[3-5]. Nucleic acid contamination from extraction reagents continues to be a common issue in today's laboratory and a popular topic for the NanoDrop technical support group.

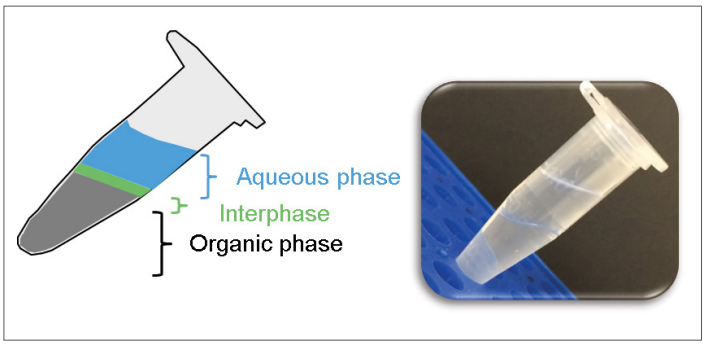

Figure 2: Phase separation typically observed when performing a conventional phenol-based nucleic acid extraction. The picture shows how difficult it is to see the phase separation.

Phenol contamination can have the following effects on nucleic acid samples:

- Phenol can effectively denature proteins, thus affecting downstream enzymatic steps.
- Phenol has a very high extinction coefficient at 270 nm; even small amounts of contamination can drastically overestimate sample concentration.

Calculating and assessing an A260/A280 (260/280) or A260/A230 (260/230) ratio is the traditional method to detect phenol contamination. However, this is not a very effective method because phenol has an absorbance peak at 270 nm, and the purity ratios of pure phenol are close to the ratios observed for pure DNA and RNA (Table 1). In this technical note, we show how different amounts of phenol contamination affect the 260/280 or 260/230 purity ratios and nucleic acid quantification results.

We also show how the Acclaro Contaminant ID feature can accurately identify phenol contamination, subtract the phenol absorbance contribution from the original measurement value, and deliver an accurate nucleic acid concentration result.

Materials and methods

Stocks of DNA and phenol were prepared as follows:

- A double stranded DNA (dsDNA) stock was prepared by diluting a salmon sperm DNA solution (Invitrogen, #15632-011) in Tris-EDTA (TE) buffer (Fisher BioReagents, pH 7.6, BP-2474-500).
- A phenol stock was prepared by diluting a buffer-saturated phenol (Fisher BioReagents, BP-1750) in TE buffer.

The concentrations of the stocks were determined on the NanoDrop One spectrophotometer against a TE blank. Nine mixtures of DNA and phenol were prepared by adding various amounts of the DNA and phenol stocks to generate the mixtures shown in Table 2.

Mixture	Target DNA Concentration	Phenol Concentration
1	225.0 ng/µL	0.00 ppm
2	225.0 ng/µL	18.75 ppm
3	225.0 ng/µL	37.50 ppm
4	225.0 ng/µL	75.00 ppm
5	225.0 ng/µL	150.00 ppm
6	225.0 ng/µL	300.00 ppm
7	225.0 ng/µL	600.00 ppm
8	225.0 ng/µL	1200.00 ppm
9	225.0 ng/µL	1600.00 ppm

Table 2: dsDNA and phenol stocks were mixed to yield the samples listed above.

Five replicates of each solution were measured on the NanoDrop One instrument against a TE blank. A fresh 1.5 µL aliquot of the appropriate mixture was used for each replicate. The dsDNA application calculated concentrations (original-uncorrected and corrected) and provided contaminant identity data, which were then used to generate the data sets presented in Table 3 for:

- Average concentrations and standard deviation (SD) based on uncorrected concentrations
- Average concentrations and SD based on Acclaro-corrected concentrations
- Average purity ratios for each mixture

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Mixture	1	2	3	4	5	6	7	8	9
Phenol content (ppm)	0.00	18.75	37.50	75.00	150.00	300.00	600.00	1200.00	1600.00
Original (Uncorrected) DNA conc ng/µL	245.75	253.50	264.34	284.75	318.35	387.55	523.59	851.29	1041.50
Corrected DNA conc ng/µL	*240.56	*238.67	240.61	243.26	244.38	242.51	245.26	265.96	265.45
Corrected DNA conc ng/µL Std dev	0.61	0.95	0.79	1.24	0.76	0.85	0.87	5.47	3.26
260/280 Purity Ratio	1.89	1.85	1.83	1.79	1.75	1.70	1.71	1.53	1.54
260/230 Purity Ratio	2.43	2.38	2.32	2.27	2.19	2.11	2.08	1.89	1.94
Acclaro Flag	No Flag	No Flag	⚠	⚠	⚠	⚠	⚠	⚠	⚠

Table 3: The DNA concentration of each mixture and DNA-only control (mixture 1) were determined with the NanoDrop One spectrophotometer using the dsDNA application. The corrected DNA concentration for mixtures 3 through 9 was obtained directly from the Acclaro contaminant analysis screen. The Acclaro Contaminant ID icon (⚠) denotes mixtures that have levels of phenol contamination high enough to trigger an Acclaro result.

The uncorrected concentrations versus the Acclaro-corrected concentrations were compared, and results are discussed below. *Mixtures 1 and 2 did not contain high enough concentrations of phenol to trigger an Acclaro result, therefore, the corrected DNA concentrations for these mixtures were determined by performing the Acclaro spectral analysis algorithm using the Thermo Scientific™ TQ Analyst™ software package.

Results and discussion

Table 3 presents the Acclaro Contaminant ID data obtained for the nine DNA/phenol mixtures described in Table 2. Notice that the phenol component is expressed in parts per million (ppm). This conversion is needed because the molar extinction coefficient of phenol is much larger than the extinction coefficient of dsDNA. For example, a 0.1% solution of phenol is 1000 ppm. As the level of phenol increases from 37.5 ppm to 1600 ppm, the discrepancy between the corrected and the uncorrected values increases. This clearly demonstrates how even very small amounts of phenol contamination can inflate an A260 concentration result. The Acclaro-corrected results demonstrate the effectiveness of the software algorithm in identifying phenol contamination, properly correcting the concentration values, and providing a more accurate concentration result than the A260 value alone.

The bar graph shown in Figure 3 compares the uncorrected and the corrected DNA concentration data in the presence of different levels of phenol contamination. It is clear that the presence of phenol inflates the concentration value significantly. The Acclaro feature flags samples when phenol contamination is greater than ~18.75 ppm, thus illustrating the high sensitivity of the software at detecting phenol contamination. The bar graph also shows that in all cases, the Acclaro-corrected DNA concentrations are within 10% of the concentration of the DNA-only control. Moreover, the concentration results were highly reproducible at high levels of phenol contamination, with average standard deviations under 5 ng/µL.

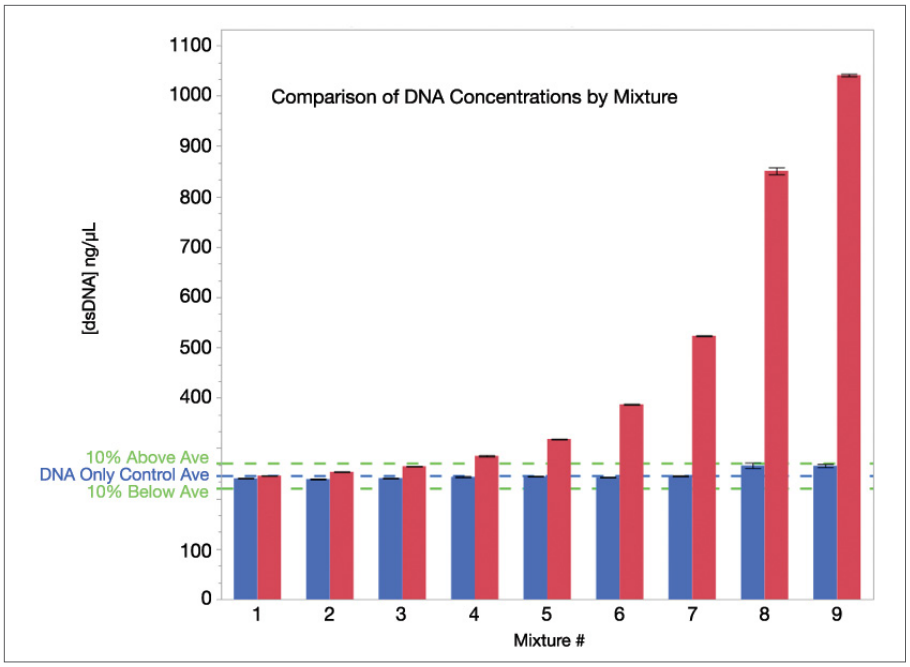

Figure 3: The DNA concentration after Acclaro correction is within 10% of the actual DNA concentration (DNA-only control) for all mixtures. Red bars represent the uncorrected DNA concentrations. The blue bars represent the corrected DNA concentrations reported by the Acclaro software (or as described in Material and Methods). The blue line is the average concentration for the DNA-only control (Mixture #1: 245.75 ng/µL without phenol). The green dotted lines represent 10% above or below the DNA-only control. Each data point represents the average of five measurements. Error bars represent one standard deviation from the mean.

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The graph in Figure 4 illustrates that increasing amounts of phenol contamination have a very minor effect on both the 260/280 and the 260/230 ratios. A DNA sample with a phenol contamination of 600 ppm has a 260/280 ratio of 1.71 and a 260/230 ratio of 2.08; these values are within the range of what is generally accepted for pure nucleic acid samples. However, with this level of phenol contamination, the DNA concentration result is generally off by greater than two-fold. Our results support the importance of not relying solely on the 260/280 and 260/230 ratios to assess the purity of a nucleic acid sample or detect contamination.

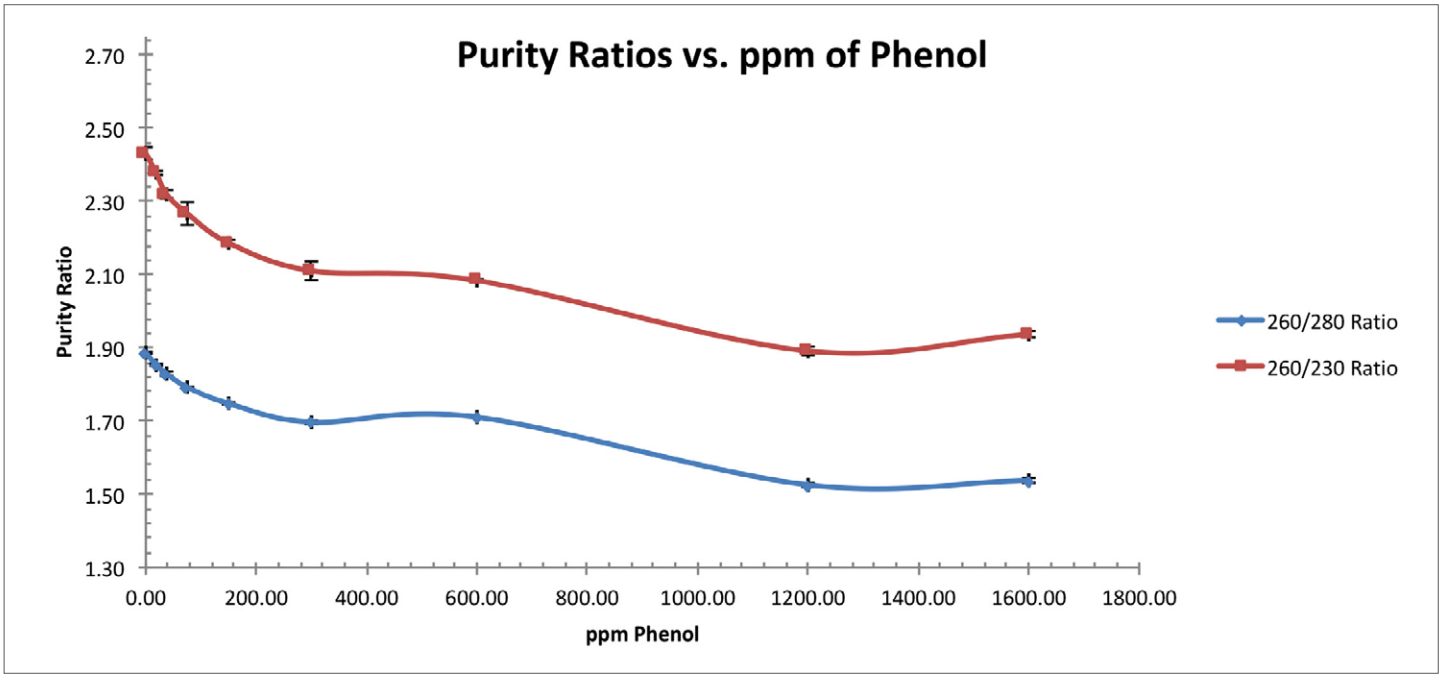

Figure 4: Average purity ratios were plotted for each dsDNA/phenol mixture. The red line denotes the 260/230 purity ratio, and the blue line denotes the 260/280 ratio.

Conclusion

The trend toward complex and demanding high-throughput genomic workflows such as qPCR, next-generation sequencing, short tandem repeat (STR) analysis, and digital PCR require rigorous quality control checks for the input nucleic acid used in these workflows. These workflows require that the concentration and purity of the sample are known and pass quality control checks. UV measurements are commonly used to determine the concentration of nucleic acid samples prior to setting up genomic workflows.

UV absorbance is quick and reliable, and it is the only quantification method that provides purity information (spectra, A260/A280 and A260/A230). Contaminants from nucleic acid extraction kits (e.g., phenol) absorb UV light in the same region of the UV spectrum as nucleic acids. The presence of these contaminants can lead to an inflated nucleic acid concentration.

Traditionally, researchers have relied on the 260/280 and 260/230 ratios as an indication of the presence of contaminants in nucleic acid solutions. Although purity ratios can be indicative of the presence of some contaminants, they do not provide definitive information about the identity or amount of contaminant.

The Acclaro sample intelligence technology built into the NanoDrop One spectrophotometer provides a chemometric approach for contaminant identification using UV spectrum analysis. In this application note, we were able to show that the Acclaro Contaminant ID feature:

- Accurately identifies phenol contamination present in nucleic acid samples
- Accurately calculates the amount of phenol contamination
- Provides corrected nucleic acid concentration values that are more accurate than using absorbance at 260 nm alone

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TECHNICAL NOTE

No. 52853

Acclaro Protein Contaminant ID

Detection of Protein in Nucleic Acid Samples Using the NanoDrop One Spectrophotometer

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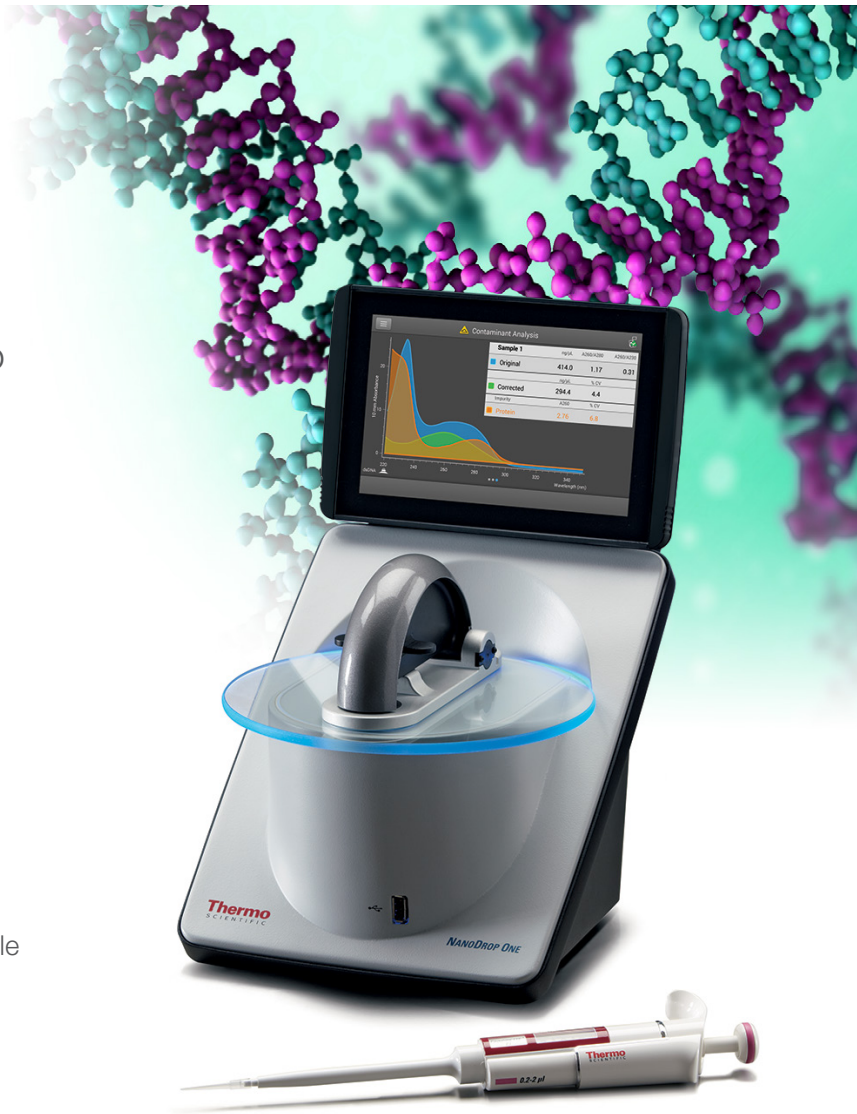

Key Words
NanoDrop One, Acclaro Contaminant ID, Chemometrics, Contaminant Identification, DNA, dsDNA, Nucleic Acid, Protein Contamination, Purity Ratio Quantification, Quantitation, Spectral Analysis, Spectrophotometer, UV-Vis

Abstract
The Thermo Scientific™ NanoDrop™ One microvolume UV-Vis spectrophotometer enables research scientists to accurately quantify nucleic acid or protein samples in the presence of common contaminants. Built with the novel Thermo Scientific™ Acclaro™ Sample Intelligence technology, the NanoDrop One instrument provides more information about sample quality by identifying common contaminants and delivering true sample concentrations. This information enables scientists to make more informed decisions as to how to proceed with downstream experiments and also provides valuable information for troubleshooting problematic extractions. Here we describe how the Acclaro Contaminant Identification (ID) feature detects protein contamination in nucleic acid samples.

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Introduction

The quantification of nucleic acids has traditionally been performed by absorbance measurements at 260 nm (Figure 1). This technique is the method of choice in the molecular biology laboratory, primarily because of the simplicity and ease by which scientists can obtain concentration and purity information about their nucleic acid samples. A consideration when using UV absorbance to evaluate samples is that many contaminants from the nucleic acid extraction process also absorb in various regions of the UV spectrum (Figure 2). Contaminant absorption within the same UV range as nucleic acids can directly affect the quantification result in two ways: it can artificially inflate the A260 value, which results in an inaccurate concentration, and it can affect the purity ratios. For a long time, purity ratios were the main way for researchers to assess the presence of UV-absorbing contaminants. However, relying on purity ratios alone does not provide a complete assessment of the potential contaminants in nucleic acid samples. Purity ratios used in combination with full-spectral data greatly enhance the ability of researchers to determine nucleic acid sample purity and ensure that an accurate concentration can be obtained using an A260 measurement.

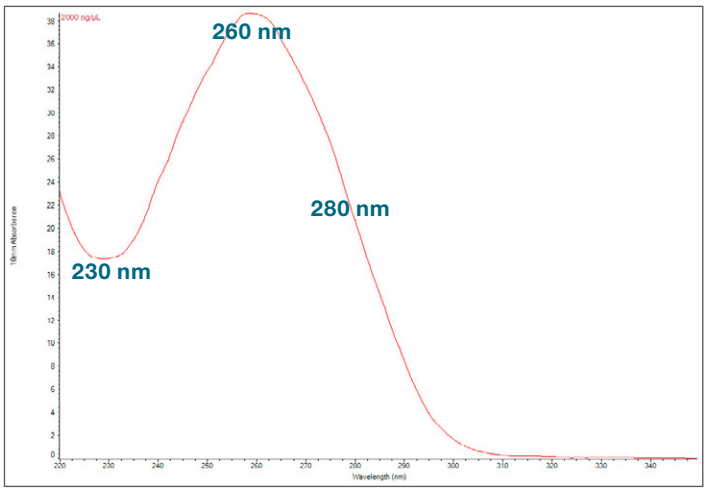

Figure 1: The UV absorbance spectrum of a pure nucleic acid sample has a peak at 260 nm and a trough at 230 nm.

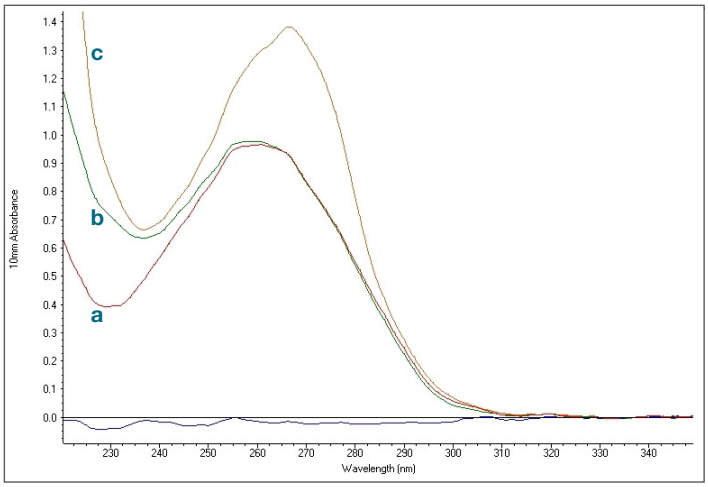

Figure 2: Contaminants can affect the UV absorbance spectrum of a nucleic acid preparation. UV spectrum of a) a pure nucleic acid sample, b) a nucleic acid sample contaminated with guanidine, and c) a nucleic acid sample contaminated with phenol.

In general, researchers will verify that their purity ratios fall into an acceptable range (Table 1). When purity ratios do not fall within the accepted range, the researcher will visually analyze the sample spectrum or seek technical assistance. Until now, analysis of a sample's spectrum has been purely qualitative, and the ability to identify specific contaminants from the spectrum has relied mostly on the experience of the researcher.

Samples	260/280	260/230
DNA	1.8–2.0	1.8–2.2
RNA	2.0–2.2	1.8–2.2

Table 1: Generally accepted purity ratio ranges for "pure" nucleic acid samples in TE buffer.

The Acclaro sample intelligence technology built into the NanoDrop One spectrophotometer provides a quantitative method for contaminant identification by using a chemometric approach to analyze the chemical components present in a sample. Acclaro software uses algorithms that rely on a reference library of spectra. These algorithms are then applied to the sample spectrum, and the software can make predictions about the presence and identity of contaminants by using chemometric mathematical principles. The Acclaro contaminant ID feature can detect protein, phenol, and guanidine salts in dsDNA and RNA samples. The NanoDrop One spectrophotometer alerts users to the presence of a contaminant in real time by displaying the yellow contaminant ID icon (shown in Figure 3a) next to the sample number. Tapping on the contaminant icon reveals the full Acclaro contaminant analysis details (Figure 3b). This screen displays the deconvolved spectra, identified contaminants, corrected DNA concentration, and a %CV, which represents the confidence of Acclaro's algorithms prediction. In this technical note, we present data that illustrates how the Acclaro sample intelligence technology detects protein contamination in nucleic acid samples.

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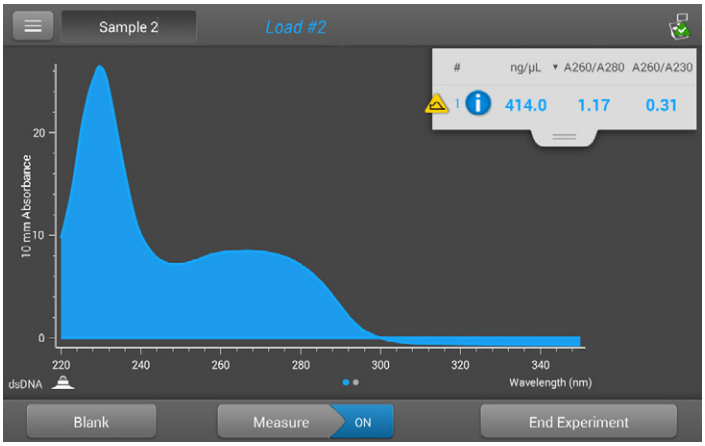

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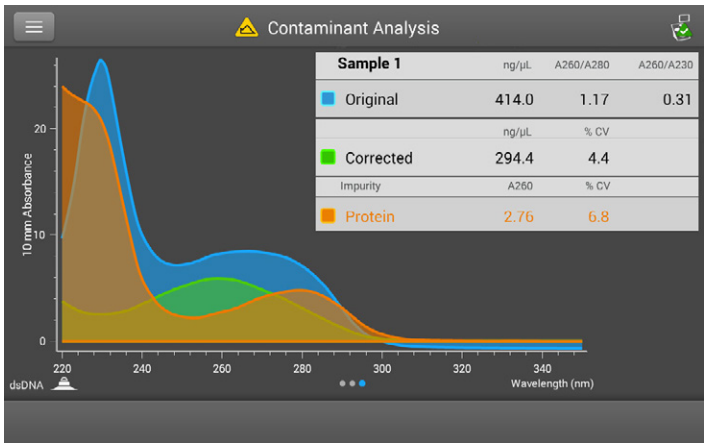

Figure 3: Acclaro Contaminant ID feature alerts the user to possible contaminants present in the sample just measured. 3a) *Measurement screen:* the Acclaro Contaminant ID icon indicates that Acclaro algorithms have detected a contaminant in this dsDNA sample. 3b) *Contaminant Analysis screen*: shows the absorbance spectra of the Original (DNA plus contaminant), Corrected (DNA minus contaminant) and Impurity (identified contaminant) and includes data on concentration, 260/280 and 260/230 ratios. To ensure reproducible results, the Corrected concentration value should be used in planning downstream experiments.

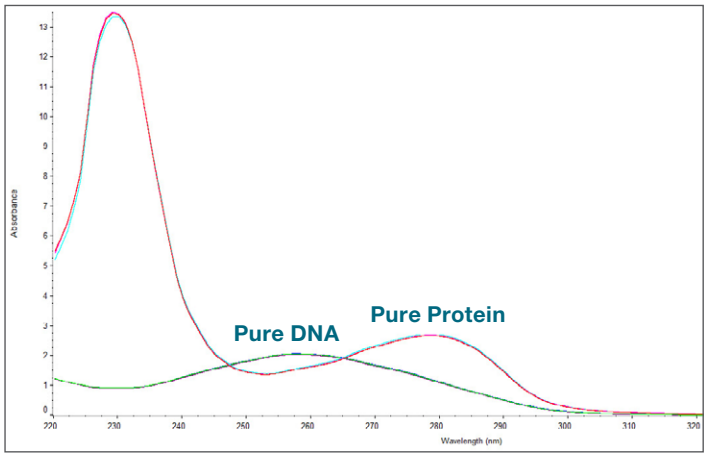

Figure 4: Pure DNA and pure protein spectra overlaid on the same graph. The DNA spectrum (green-blue) has the characteristic peak at 260 nm and trough at 230 nm, whereas, the protein spectrum (red-blue) has the characteristic peak at 280 nm and an increase in absorbance below 250 nm.

Protein Contaminants in Nucleic Acid Samples

Protein as a contaminant in nucleic acid preparations 1) contributes to the absorbance at 260 and inflates the concentration of the nucleic acid and 2) is usually detected by a decrease in the 260/280 purity ratio. The decrease in this ratio occurs because the amino acid residues tryptophan, tyrosine, phenylalanine, as well as the Cystine disulfide bonds absorb light at 280 nm (Figure 4). Most nucleic acid extraction kits ensure an adequate removal of protein; however, many researchers new to phenol/chloroform extractions will often experience protein contamination. During this type of extraction, researchers have to separate the aqueous phase from the organic phase. Because proteins precipitate at the interface between these two phases, it is easy to accidentally bring proteins from the interface into the aqueous phase when performing the nucleic acid extraction, thus introducing protein contamination into their nucleic acid preparation.

The 260/280 ratio was originally used as a very sensitive way to detect DNA contamination in protein preparations (Warburg, 1942).[1] The molecular biology community later adopted the 260/280 ratio as a way to detect protein contamination in nucleic acid preparations. However, the 260/280 ratio, as a means for detecting protein contamination in nucleic acid samples, has its limitations. The extinction coefficients of proteins are very small relative to those of nucleic acids and, therefore, it takes a large amount of protein to affect the 260/280 purity ratio (Glasel, 1995, Huberman, 1995, and Manchester, 1995).[2-4] Nonetheless, scientists still encounter nucleic acid preparations with low 260/280 purity ratios. Protein contamination will not only mislead researchers by inflating the nucleic acid concentration result, but it can also directly affect downstream reverse transcription and qPCR reactions by inhibiting or interfering with enzymatic reactions. In this technical note, we show how the degree of protein contamination affects the purity ratios and quantification results. We also show that the Acclaro Contaminant ID feature of the NanoDrop One spectrophotometer can accurately identify protein as the contaminant and deliver accurate nucleic acid concentration results.

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Materials and Methods

Stocks of DNA and protein were prepared as follows. The double-stranded DNA (dsDNA) stock was prepared by diluting a salmon sperm DNA solution (Invitrogen™, #15632-011) in Tris-EDTA (TE) buffer (Fisher BioReagents™, pH 7.6, BP-2474-500). The protein stock was prepared by diluting a solution of bovine serum albumin (BSA, Sigma Aldrich®, #A7284) in TE buffer. The concentrations of both dsDNA and BSA stocks were determined on the NanoDrop One spectrophotometer against a TE blank. Mass calculations were made using the factor 50 ng-cm/µL for dsDNA and the extinction coefficient E1% 6.7 for BSA. Nine mixtures of DNA and protein were then prepared by adding various amounts of the DNA and protein stocks to generate the mixtures shown in Table 2.

Five replicates of each solution 1–9 were measured on the NanoDrop One spectrophotometer against a TE blank. A fresh 1.5 µL aliquot of the appropriate mixture was used for each replicate. The software calculated concentrations (corrected and original/uncorrected) and Acclaro Contaminant identity data, were then used to generate the data sets presented in Table 3:

- Average concentrations and standard deviation (SD) based on uncorrected concentrations
- Average concentration and SD based on Acclaro corrected concentrations
- Average purity ratios for each mixture

The original (uncorrected) concentrations versus the Acclaro-corrected concentrations were compared, and results are discussed below. Since mixtures 1–4 did not contain high enough protein levels to trigger an Acclaro result, the corrected DNA concentration for mixtures 1 through 4 were determined by performing the Acclaro spectral analysis using the Thermo Scientific™ TQ Analyst™ software package.

Mixture	%DNA (by mass)	% Protein (by mass)
1	100.0	0.0
2	57.1	42.9
3	40.0	60.0
4	28.6	71.4
5	16.7	83.3
6	10.0	90.0
7	7.1	92.9
8	4.8	95.2
9	1.6	98.4

Table 2: DNA and protein stocks were mixed to yield the proportions shown above.

Mixture	1	2	3	4	5	6	7	8	9
% Protein (by weight) in Mixture	0.0	42.9	60.0	71.4	83.3	90.0	92.9	95.2	98.4
Original (Uncorrected) DNA [conc] ng/µL	531.8	534.7	542.1	571.5	591.3	644.4	672.9	740.4	1097.6
Corrected DNA [conc] ng/µL	526.8	523.9	525.9	540.4	541.1	554.6	549.2	547.3	560.1
260/280 Purity Ratio	1.94	1.89	1.84	1.74	1.60	1.45	1.34	1.21	0.89
260/230 Purity Ratio	2.45	1.67	1.34	0.90	0.61	0.41	0.32	0.26	0.19
Acclaro Flag	No	No	No	No	⚠	⚠	⚠	⚠	⚠

Table 3: The uncorrected (original) DNA concentration of each mixture was determined with the NanoDrop One spectrophotometer using the dsDNA application. The corrected DNA concentration for mixtures 5–9 was obtained directly from the NanoDrop One Acclaro contaminant analysis results. The Acclaro contaminant ID icon(⚠) denotes mixtures that have levels of protein contamination high enough to trigger an Acclaro result.

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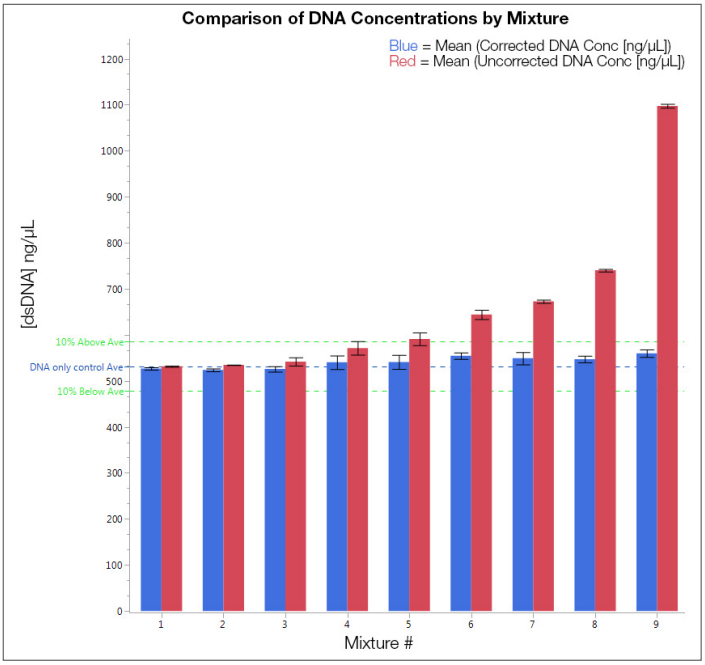

Figure 5: The Acclaro-corrected concentration is within 10% of the actual DNA concentration for all mixtures. Red bars represent the uncorrected (original) DNA concentrations. The blue bars represent the corrected DNA concentrations reported by Acclaro software (or calculated for mixtures 1–4 as described in Materials and Methods). The dotted blue reference line is the concentration for the DNA-only control (mixture #1 – the actual dsDNA concentration). The dotted green reference lines represent either 10% above or below the DNA-only control average, 531.8 ng/µL. Each data point represents the average of five measurements. Error bars represent 1 standard deviation from the mean.

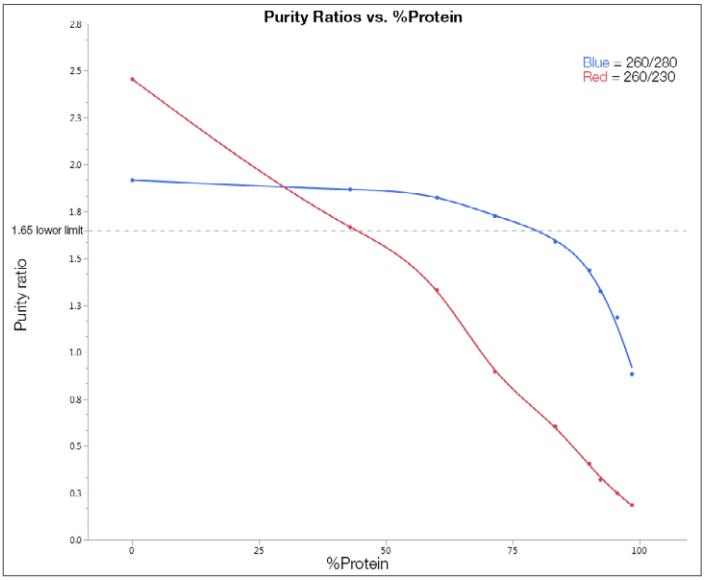

Figure 6: In this graph the average purity ratios are plotted for each dsDNA/Protein mixture (mixtures 1–9). The red line denotes the 260/230 purity ratio, and the blue line denotes the 260/280 ratio.

Results and Discussion

Table 3 presents the Acclaro Contaminant ID data obtained for the nine DNA/protein mixtures described in Table 2. As the level of protein contamination increases, the discrepancy between the corrected and the original (uncorrected) results becomes larger. This emphasizes how protein contamination can inflate an A260 concentration result. The Acclaro-corrected results show how the software algorithm can quantitatively correct for these levels of protein contamination and provide a more accurate DNA concentration than the A260 value alone. The data in Table 3 also demonstrate that a large amount of protein contamination is required before a significant change in the 260/280 purity ratios is observed. Samples with as much as ~72% protein by weight still have an acceptable 260/280 purity ratio. As the protein contaminant levels increase from ~72% to 98%, the 260/280 ratio drops steadily from 1.74 to 0.89.

The bar graph in Figure 5 shows a comparison between the uncorrected and corrected DNA concentration data in the presence of different levels of protein contaminant. The presence of protein can inflate the original reported concentration. The data also shows that when the contaminant concentration is above ~72% protein by weight, Acclaro flags the sample and displays a corrected concentration (Table 3). The corrected DNA concentrations are within 10% of the result for the DNA-only control. Mixture 9, which has the highest amount of protein contamination, shows the largest difference between the corrected and uncorrected concentration results. However, even with this extremely contaminated mixture where the protein represents >98% of the analyte mass in the sample, the Acclaro-corrected result brings the concentration result for mixture 9 to within 10% of the actual DNA concentration. These results were highly reproducible with standard deviations averaging under 1 ng/µL.

The graph in Figure 6 shows how purity ratios change with increasing levels of protein. As expected, as the percentage of protein contamination increases, the 260/280 purity ratio decreases. However, as shown in Figure 6, the amount of protein has to be larger than 75% of the sample to observe a significant decrease in the 260/280 purity ratio (below 1.65), which is the lower limit generally accepted for use in downstream experiments. On the other hand, the 260/230 purity ratio steadily decreases as the percentage of protein in the mixture increases. Figure 6 indicates that the

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260/230 ratio may be a more sensitive indicator of protein contamination. The challenge of using this ratio to detect protein contamination is that many other common contaminants, such as common salt buffers, guanidine salts, and polysaccharides, can affect this ratio as well. Therefore, the 260/230 ratio alone cannot confirm protein contamination. The Acclaro Contaminant ID feature of the NanoDrop One spectrophotometer delivers a definitive advantage to the researcher by providing a corrected DNA concentration and by identifying contaminants present in a nucleic acid sample.

Conclusion

Experiments that use nucleic acids require that the concentration and purity of the sample is known. The UV-Vis method used for the quantification of nucleic acid preparations relies on the absorbance of nucleic acid molecules at 260 nm to determine the concentration of nucleic acids in solution. Contaminants such as proteins that are co-purified with nucleic acids can also absorb light in the UV region of the spectrum, which leads to overestimating the calculated nucleic acid concentration.

Traditionally, researchers have relied on purity ratios as an indication of the presence of contaminants in nucleic acid samples. “Out of range” purity ratios can inform users of the presence of contaminants, but they do not provide information on the identity and amount of the contaminant present. The Acclaro sample intelligence technology in the NanoDrop One spectrophotometer provides a chemometric approach for contaminant identification using UV spectrum analysis. This feature empowers researchers by helping them 1) identify the type of contaminant present in their sample 2) determine the level of contamination and 3) obtain a corrected nucleic acid concentration. By using the Acclaro sample intelligence technology, researchers are now able to make informed decisions on how to troubleshoot sample preparations to reduce contamination and how to proceed using a sample in downstream experiments.

In this application note, we show how the Acclaro Contaminant ID feature:

- Accurately identifies protein as the contaminant present in nucleic acid samples
- Accurately calculates the amount of protein contamination present in a nucleic acid sample
- Provides corrected concentration results that are more accurate than using absorbance at 260 nm alone. Even at very high levels of protein contamination, the corrected values are within 10% of the actual nucleic acid concentration.

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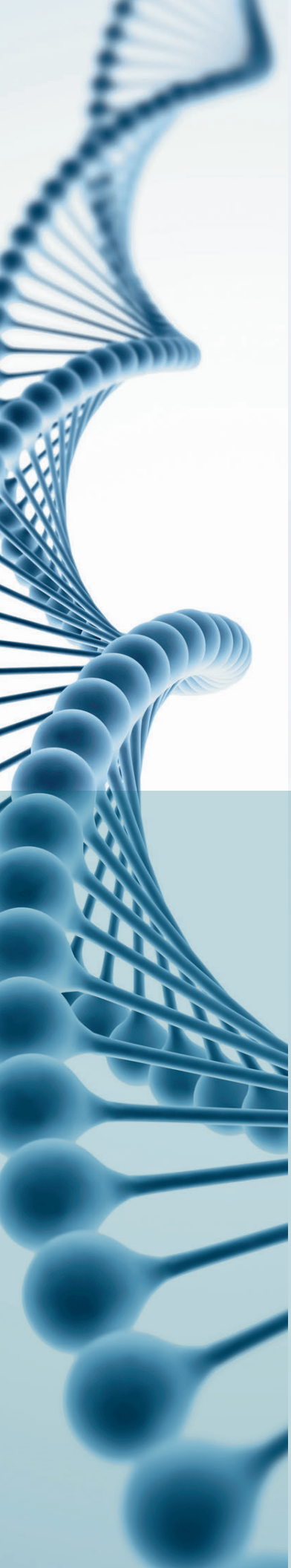

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