Application note

Impact of contaminants on nucleic acid fluorescence measurements

Introduction

Quantification of nucleic acids after their extraction from source materials is often performed using UV-Vis absorbance at 260 nm or fluorescent dye binding assays. For high sensitivity, fluorescence is generally preferred over UV-Vis absorbance. However, contaminants such as salts, phenol, or proteins are frequently co-extracted, and absorbance purity ratios, specifically A260/A280 and A260/A230, provide insight into contamination presence.[1-3] It is well characterized that contaminants can inhibit polymerase chain reactions (PCR), underscoring the need of a purity checkpoint prior to downstream workflows.[1-5] For nucleic acid fluorescent dye binding assays, a variety of detergents, salts, and organic solvents as well as changes in pH have been shown to perturb dye binding at certain concentrations.[6-9] Herein, the impact of contaminants on the Thermo Scientific™ NanoDrop™ Ultra dsDNA Broad Range (BR) Fluorescence Assay Kit, as well as the role of UV-Vis absorbance in assessing sample quality, were evaluated.

Methods

Lambda dsDNA (Thermo Scientific™, SD0011) was diluted in tris-EDTA (TE) buffer and contaminants were spiked as listed in Table 1. Final dsDNA concentrations were 100, 50, 10 and 0 ng/µL, where each concentration was mixed with the low, medium, and high levels of contaminants.

For absorbance, nucleic acid samples were measured using the microvolume pedestal on a Thermo Scientific™ NanoDrop™ Ultra^{C} FL Microvolume Spectrophotometer and Fluorometer using the dsDNA application. The NanoDrop instrument was blanked with TE buffer and measurements were performed in replicates of five. Fluorescence was performed using the NanoDrop Ultra dsDNA BR assay kit per the manufacturer's instructions. Measurements were taken in replicates of five on the NanoDrop Ultra^{C} FL instrument using the microvolume pedestal and the blue LED.

Contaminant level	BSA	Phenol	Ethanol	Sodium acetate
Low	0.6 mg/mL	0.5%	1%	10 mM
Medium	1.0 mg/mL	1%	15%	50 mM
High	2.0 mg/mL	2%	30%	100 mM

Table 1. Final concentrations of contaminants spiked in dsDNA preparations.

Results

To confirm any differences in absorbance and fluorescence readings could be attributed to contamination presence, nucleic acid controls were compared (Figure 1). The concentration results of the controls were well correlated, indicating strong agreement between the two methods.

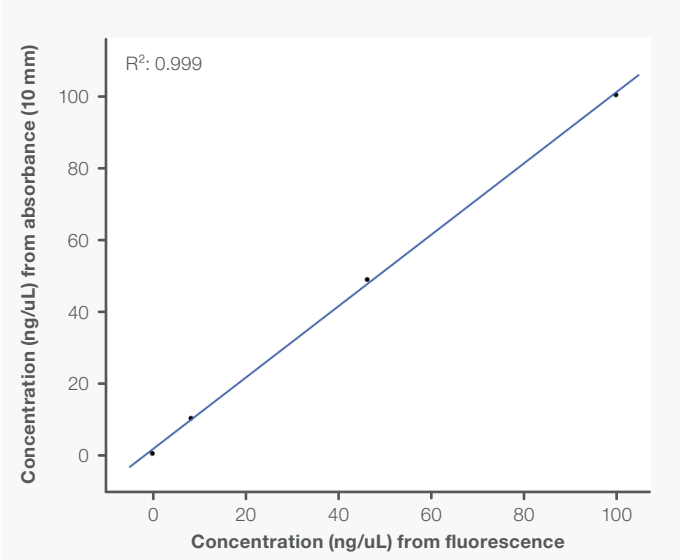

Figure 1. Linearity comparison of concentration of lambda dsDNA controls determined by absorbance and fluorescence using the NanoDrop Ultra^{C} FL spectrophotometer and fluorometer. Points represent the mean (n=5); R^2=0.999.

The pure absorbance spectra of each contaminant, along with a 100 ng/µL dsDNA control, are displayed in Figure 2. All tested contaminants—phenol, protein (BSA), ethanol, and sodium acetate—exhibited absorbance at and below 230 nm, while ethanol, phenol, and protein (BSA) also showed absorbance at 260 nm and 280 nm. Because nucleic acid quantification by UV-Vis spectrophotometry utilizes absorbance at 260 nm, the presence of contaminants that have overlapping spectral profiles leads to overestimation of nucleic acid concentration.

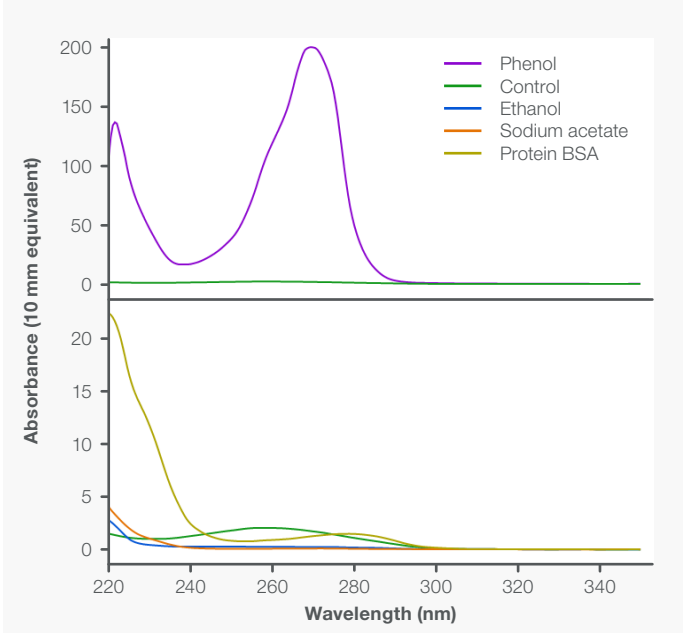

Figure 2. Spectra overlay of pure stock contaminants blanked with dH_2O and dsDNA blanked with TE buffer. Top: 100 ng/µL control dsDNA (green) and phenol (purple). Bottom: 100 ng/µL control dsDNA (green), protein BSA (yellow), ethanol (blue), and sodium acetate (orange).

Reviewing the absorbance spectrum and purity ratios (A260/A230, A260/A280) of a nucleic acid sample provides insight into contaminants that are present. Table 2 outlines the relationship between purity ratios and the increasing amounts of contaminants. Acceptable A260/A280 and A260/A230 purity ratios for "pure" dsDNA are 1.8 and 2.0–2.2, respectively. Deviations in purity ratios are related to the absorbance profile of contaminants, with protein contamination mainly affecting A260/A280 while organic solvent and salt contamination affect A260/A230.

Contaminant level	Low			Medium			High		
Purity ratios & FL percent error	**A260/ A280**	**A260/ A230**	**% Error**	**A260/ A280**	**A260/ A230**	**% Error**	**A260/ A280**	**A260/ A230**	**% Error**
Control (no contaminant)	1.86	2.08	1.53	-	-	-	-	-	-
BSA	1.58	0.6	**7.48**	1.41	0.38	**24.04**	1.17	0.24	**31.08**
Phenol	2.17	31.8	**11.05**	2.45	2.59	**12.01**	2.34	2.53	**17.32**
Ethanol	1.89	2.12	**17.19**	1.87	2.28	**17.07**	1.85	2.58	**31.33**
Sodium acetate	1.92	4.21	**44.9**	1.92	4.26	**43.79**	1.9	2.75	**58.15**

Table 2. Absorbance purity ratios (A260/A280 and A260/A230) and fluorescence percent error for the 100 ng/µL dsDNA sample per contaminant type and spike level. Red values indicate outside of acceptable range, green values are within acceptable range. Percent error cutoff is 20% per the acceptance criteria of the NanoDrop Ultra dsDNA BR assay kit.

To provide support in determining sample purity, the Thermo Scientific™ Acclaro™ Contaminant Identification (ID) Technology detects the presence of protein and phenol in dsDNA samples. This built-in feature for all NanoDrop Ultra models analyzes each sample spectrum and utilizes chemometric algorithms to predict contaminant identity. Detecting contaminants is critical, as they can directly influence fluorescence quantification accuracy as demonstrated in Table 2. As the purity ratios deviated from the acceptable criteria, fluorescence percent error increased with higher levels of protein (BSA), phenol, ethanol, and sodium acetate contamination. This finding was consistent across the dsDNA concentration range, as illustrated in Figure 3 for 50 ng/µL dsDNA. These results demonstrate that fluorescent dye binding assays are not inherently protected from contamination, and an absorbance purity measurement is crucial for accurate quantification.

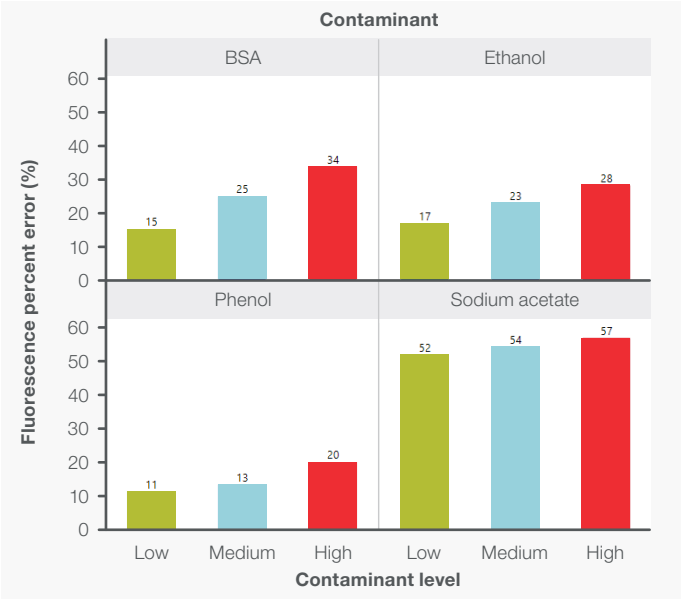

Figure 3. Relationship between fluorescence concentration percent error (%) and contaminant level for the 50 ng/µL dsDNA concentration dataset. Bars represent the mean percent error (n=5) for contaminant levels high (red), medium (blue), and low (green).

The concentrations reported by absorbance and fluorescence are outlined in Figure 4. Absorbance by UV-Vis spectrophotometry is a direct, additive measurement; any material absorbing light at the analytical wavelength will contribute to the overall absorbance and concentration. For nucleic acids, this wavelength is 260 nm and the absorbance concentration results are inflated with increasing levels of contamination by protein (BSA), phenol, and ethanol, which correlate with their absorbance profiles. Using phenol as an example, the spectrum in Figure 2 is absorbing at 260 nm approximately fifty-fold greater than the dsDNA control. This is due to phenol's high extinction coefficient; a small amount of phenol is needed to artificially inflate nucleic acid concentration via UV-Vis spectrophotometry.

By contrast, quantifying nucleic acids via fluorescence relies on dye binding. The data presented in Figure 4 suggest fluorescent measurements were impacted by the presence of protein (BSA), phenol, ethanol, and sodium acetate. The fluorescent signal decreased as contaminant concentration increased, indicating dye-to-nucleic acid interactions are sensitive to contaminants in a dose-dependent manner. While the specific mechanism by which contaminants impact fluorescent signals is out of scope for this note, the trend indicates that co-extracted contaminants can introduce errors into both absorbance and fluorescence-based quantification.

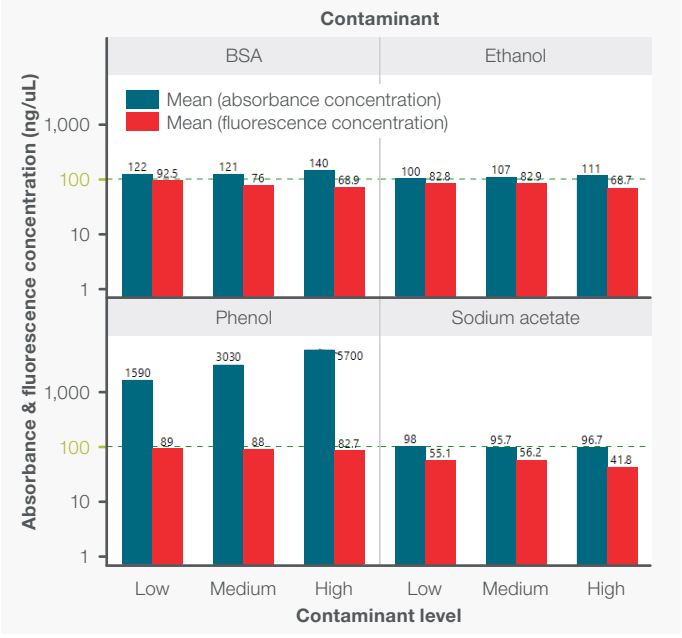

Figure 4. Comparison of absorbance (blue bars) and fluorescence-based (red bars) quantification for the 100 ng/µL dsDNA sample in the presence of increasing levels of contaminants. Bars and labels represent the mean concentration (n=5). The green dashed lines indicate the control concentration at 100 ng/µL. The y-axis is displayed on a logarithmic scale.

Conclusions

Contaminants commonly co-extracted from nucleic acid preparations were shown to introduce error in both absorbance and fluorescence-based quantification. While absorbance measurements were artificially inflated by contaminants absorbing at 260 nm, the fluorescence signal was reduced in the presence of contaminants. Nucleic acid absorbance purity ratios (A260/A280, A260/A230) provided contaminant insight and were shown to predict fluorescence performance. Assessing spectral data and purity ratios, along with the Acclaro contaminant ID technology, provides information on nucleic acid quality and determines whether additional purification is required before quantification. Since the NanoDrop Ultra FL instrument is both a UV-Vis spectrophotometer and fluorometer, performing a quick quality measurement with absorbance prior to a sensitive fluorescence measurement can improve confidence in nucleic acid quantification.

References

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