Micro-Volume Purity Assessment of Nucleic Acids using A_{260}/A_{280} Ratio and Spectral Scanning

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A common method to determine the purity of biomolecules from sample isolates is by use of a spectrophotometric ratio using absorbance measurements at wavelengths of 260 nm and 280 nm. The A_{260}/A_{280} ratio provides a rapid indication of protein contamination in nucleic acid isolates and less commonly, nucleic acid contamination in protein isolates. In addition, UV-Vis scanning can identify contamination from chemical additives used in the isolation procedure. Here we assess this capability in micro-volume analysis using the Take3™ microplate accessory.

Key Words:

Protein Purity
Nucleic Acid Purity
A_{260}/A_{280} Ratio
Spectral Scanning

Introduction

The spectrophotometric analysis of biomolecules for sample quantification and purity assessment has been well documented. Quantification is made possible by the absorption of ultra-violet light by chromophores contained in nucleic acids and protein. Consistent with these methods is the use of the Beer-Lambert Law that relates physical property of light absorption by molecules to concentration within the sample by the following equation:

$$A = \log \frac{I_o}{I} = \varepsilon l c$$

where ε refers to the molar absorptivity or extinction coefficient of the analyte, l is the path length (cm) and c is the concentration of the analyte . The average extinction coefficient for dsDNA = 0.020 $(ng/\mu L)^{-1}cm^{-1}$; RNA = 0.027 $(ng/\mu L)^{-1}cm^{-1}$and protein = 0.001 $(ng/\mu L)^{-1}cm^{-1}$. Traditional UV-Vis spectrophotometry relied on vessels with a standard 1 cm pathlength for measurement simplifying the calculation where A/ε=c. Yet the process of quantification was made difficult by the high concentration of the isolated sample and the large pathlength of 1 cm vessels, which required a sample dilution step before quantification. This dilution step can be obviated by the significant reduction of path length, which spawned micro-volume analysis and is typically performed with path lengths more than an order of magnitude lower. The Beer-Lambert law will of course still hold for these lower pathlength analyses allowing for accurate quantification without sample dilution[1].

The assessment of the purity of a nucleic acid sample is often performed by a procedure commonly referred to as the A_{260}/A_{280} ratio which refers to two spectrophotometric measurements made at these defined wavelengths. For double stranded DNA, the commonly accepted average extinction coefficients at 260 nm and 280 nm is 0.020 and 0.010 $(ng/\mu L)^{-1}cm^{-1}$ respectively; similarly for proteins, the average extinction coefficient values at 260 nm and 280 nm are 0.00057 and 0.001 $(ng/\mu L)^{-1}cm^{-1}$ respectively. Thus it is obvious that nucleic acid samples would be expected to have a higher absorbance at 260 nm than at 280 nm, while for a protein sample the converse would be true. Using these extinction coefficients, pure nucleic acid samples would have an A_{260}/A_{280} ratio of 2.0, while protein would be 0.57. Samples that contain a mixture of protein and DNA would of course be influenced by both macromolecules. According to the Beer-Lambert law, the total absorbance of a solution is the sum of the absorbances of the components comprising the solution, thus in the case of a mixture of nucleic acid and protein the following equation would be true:

$$\frac{A_{260}}{A_{280}} = \frac{(\varepsilon_{DNA,260})(\%DNA+) + (\varepsilon_{Protein,260})(\%Protein)}{(\varepsilon_{DNA,280})(\%DNA) + (\varepsilon_{Protein,280})(\%Protein)}$$

Note that since a ratio is taken of absorbances, the pathlength variable is cancelled out, provided the same vessel is used for both measurements.

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In some instances additional information can be obtained by performing a spectral analysis of the sample. Typically, biomolecules are scanned between the wavelengths 220 and 300 nm at 1 nm increments. Spectra covering this range will insure capture of the absorption profiles of biomolecules as well as those of any potential contaminants, should they posses a chromophore of significant absorptivity over this wavelength range. Therefore, spectral analysis can provide a relatively quick method of detecting the presence of contaminants without destruction of the sample.

Here we present the ability to use a micro-volume vessel in conjunction with several different microplate readers for quantification of microliter volumes of biomolecules. The ability to detect common contaminants used in molecular biology techniques is also presented.

Materials and Methods

Double-stranded DNA (dsDNA) samples were prepared using herring sperm dsDNA (Cat. No. D6898) purchased from Sigma-Aldrich Corp. (St. Louis, MO) resuspended in TE (10 mM Tris, 1 mM EDTA pH 8.0) buffer. Protein samples were prepared using bovine serum albumin (BSA) (Cat. No. A3294) purchased from Sigma-Aldrich Corp and resuspended in MilliQ™ water. Concentrations depicted are based on either a 0.5 mm or a 1 cm path length and 50 µg/mL/OD for DNA and 0.667 mg/mL/OD for BSA protein.

Double-stranded DNA spectral scanning measurements were made using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader, Epoch™ Microplate Spectrophotometer or Eon™ Microplate Spectrophotometer in conjunction with a Take3™ Micro-Volume Plate (BioTek Instruments, Inc., Winooski, VT), which has a nominal pathlength of 0.5 mm, or 20x smaller than the typical 1 cm pathlength of standard spectrophotometric cuvettes. A five point 1:2 dilution series was made using a dsDNA stock solution (70 ng/µL). Sample blanks consisted of either 2 µL of TE or MilliQ water for dsDNA or protein, respectively. Samples were blanked by pre-reading the Take3 plate using the appropriate solvent at each wavelength and subtracting from the sample measurements. The blanks were removed from the Take3 using a laboratory wipe and duplicate samples of each biomolecule concentration were loaded onto to the Take3 plate using a manual pipettor.

For comparison of pure biomolecular samples versus heterogeneous samples, dsDNA, protein and heterogeneous samples were made using stocks solutions of dsDNA (100 ng/µL) and BSA (625 ng/uL). Percent accuracy was calculated for each species by comparing determinations calculated from peak absorbances at 260 or 280 nm for dsDNA and BSA, respectively, using the extinction coefficients described above, to the expected concentration of each component.

Samples for analysis of trace contamination by phenol and guanidine hydrochloride were made using a stock of dsDNA (2,000 ng/µL) spiked with increasing concentrations of contaminant.

Results and Discussion

Figure 1 consists of a series of dsDNA concentrations ranging from 6.5 to 67.5 ng/µL. The composite spectral analysis evident indicates an absorbance peak at a wavelength of ~260 nm which is typically associated with highly purified dsDNA that correlates with the increasing dsDNA concentration. This peak is discernible down to low ng/µL concentrations and can be used in conjunction with the mass extinction coefficient to calculate the concentration of DNA in the sample.

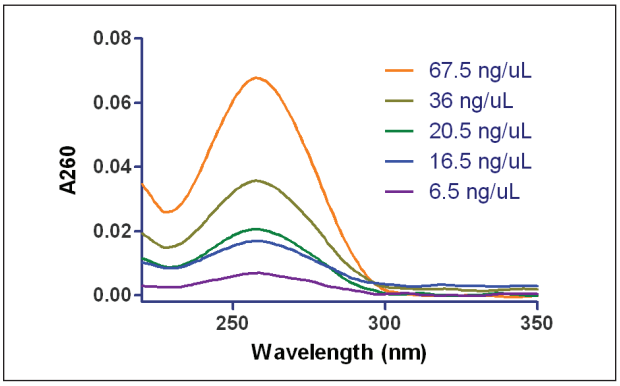

Figure 1. **Micro-volume spectral scanning analysis. Spectral scanning micro-volume analysis representing duplicate 2 µL samples of dsDNA in TE buffer using the Synergy H4 and Take3 Micro-Volume Plate. The data indicates the ability to perform micro-volume spectral analysis on sample in the low ng/µL range. (Values represent blanked data normalized to a 0.5 mm path length).**

Common contaminants that co-purify during biomolecule isolation are often of biological origin such as protein contamination of DNA or vice versa. Spectral analysis of samples can be compared to spectra of known standards for comparison by a trained eye. Standards of dsDNA, protein and a complex mixture of a 1:10 DNA/protein (w/w) was subject to spectral scanning from 230 to 290 nm for analysis (Figure 2). While these spectra were meant to present a clear example of the differences that might be seen when analyzing purified or complex samples, resultant spectra may not show obvious variances indicative of what molecules constitute the sample.

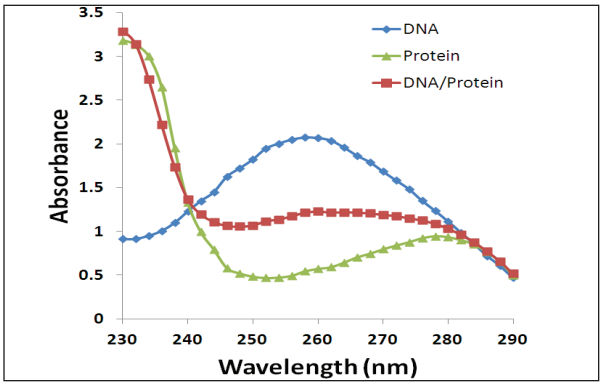

Figure 2. Biomolecule spectral data comparison. Typical absorbance profiles of purified dsDNA, BSA protein, and a complex mixture containing 1:10 DNA/protein (w/w). Spectral analysis of samples was performed at 1 nm increments from 230 nm to 290 nm. Data normalized to a 1 cm path length.

Calculating the ratios of two or more wavelengths of a spectral analysis provides a quick method to determine if a contaminant may be present in a biomolecular sample such as DNA. Significant concentration dependent differences can be observed in the spectra at 260 nm with relatively little change observed at 280 nm over a broad range of DNA/protein sample concentration ratios (Figure 3). Careful examination at the A280 ratio profiles for several samples show the expected spectral peaks at 260 nm for purified dsDNA and at 280 nm for purified protein (Figure 4). Typical A_{260}/A_{280} ratios for purified DNA and protein are 1.8 and 0.6, respectively. However, while there is a significant concentration dependent change in the A_{260} and A_{280} measurements as the ratio of sample constituents change, considerable protein contamination is required before it is reflected in the A_{260}/A_{280} ratio (Figure 5).

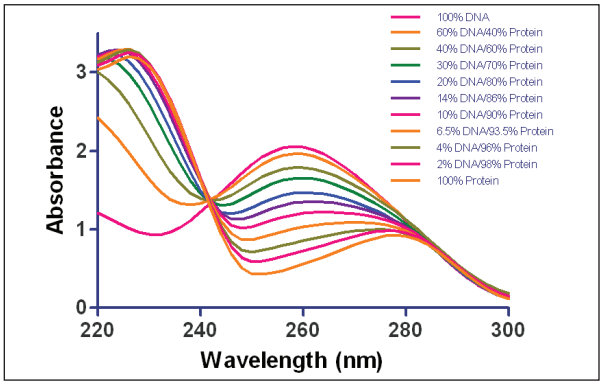

Figure 3. Absorbance spectral scans of purified dsDNA, protein or a mixture. Samples contained purified herring sperm dsDNA, BSA protein or a DNA/protein mixture at varying ratios (w/w). Measurements were taken at 1 nm intervals at wavelengths ranging from 220 to 300 nm. Data normalized to a 1 cm path length.

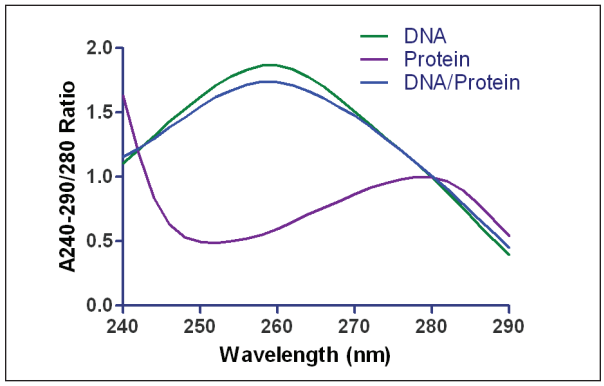

Figure 4. Typical A_{280} ratios associated with samples of biomolecules. Samples contained purified dsDNA, protein or a DNA/protein mixture (60%/40%) (w/w). Measurements were taken at 1 nm intervals at wavelengths from 240 to 290 nm. The A_{280} ratio was then calculated by dividing each wavelength measurement by the A_{280} measurement for each sample. Data has been normalized to 1 cm.

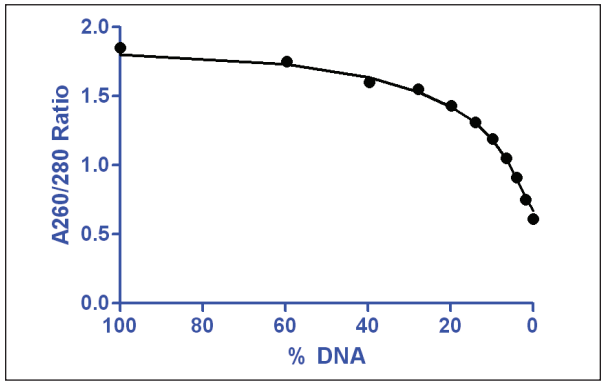

Figure 5. A_{260}/A_{280} ratios associated with increasing protein contamination of DNA. Samples contained purified herring sperm dsDNA with increasing concentrations of BSA ranging from 40-100% (w/w). Measurements were taken at 260 to 280 nm. The A_{260}/A_{280} ratio was then calculated for each DNA/protein mixture. Data has been normalized to 1 cm.

When biomolecular concentrations are calculated from absorbance measurements taken from heterogeneous samples considerable inaccuracies may occur (Table 1). Again, this effect is most notable for protein samples containing low levels of DNA contamination while the effects of DNA contamination by low levels of protein leads to relatively minor inaccuracies. Of major concern are complications that may arise during downstream processing should samples contain interfering molecules. Contaminants can cause such effect as catalytic inefficiencies due to quantification inaccuracies of necessary reaction components or substrate inhibition.

% DNA	% Protein	% Accuracy [DNA]	% Accuracy [Protein]
100.0	0.0	0.0	N/A
59.7	40.3	9.6	1107.3
39.7	60.3	18.5	445.9
27.8	72.2	29.0	285.3
19.8	80.2	44.3	189.0
14.1	85.9	62.9	121.4
9.9	90.1	91.5	86.1
6.6	93.4	139.2	57.4
4.0	96.0	226.8	28.5
1.8	98.2	498.0	15.7
0.0	100.0	N/A	0.0

Table 1. Calculated percent accuracy determinations from spectral data: DNA/protein. The accuracy of DNA and protein concentrations were calculated using peak absorbance measurements at 260 and 280, respectively. Extinction coefficients of 50 ng/µL/OD and 0.667 µg/µL/OD for DNA and protein, respectively, were used for comparison of calculated determinations to expected values and expressed as percent accuracy.

While contamination of biomolecules by competing species represents a major challenge other contaminants from processing may also be present. Contaminants such as alcohols, phenols, and salts represent common residual components from reagent kits used in the purification and manipulation of DNA and proteins. Many contaminants can interfere with accurate quantification of purified biomolecules due to similar absorbance profiles with overlapping spectral peaks. A common method to insure sample integrity when purifying nucleic acids is to treat the source sample with a denaturant to remove ubiquitous nuclease activity. Guanidine hydrochloride (GuHCl) at a stock concentration of 8M is typically added to lysis or homogenization buffers during sample preparation. Residual GuHCl at trace levels (<1.0%) can impact quantification of DNA due to an apparent decrease in the spectral absorbance peak at approximately 260 nm (Figure 6 and Table 2). This may be due to the chaotrophic effect of reagent causing partial denaturation of the DNA and appears to be independent of concentration.

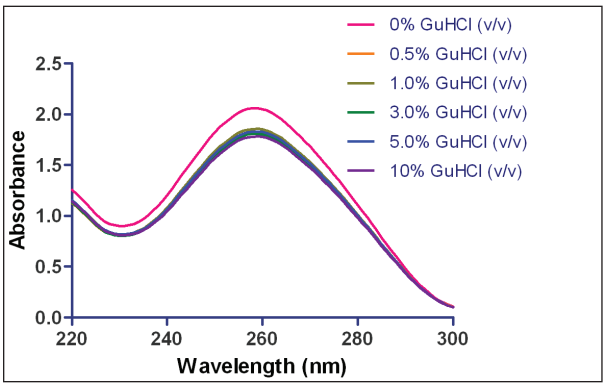

Figure 6. Spectral analysis of DNA: GuHCl contamination. Spectral analysis of herring sperm dsDNA spiked with increasing concentrations of guanidine hydrochloride. Data has been normalized to a 1 cm pathlength.

[GuHCl] ($\%^{v}/_{v}$)	A260/280	Calc'd [DNA] (% accuracy)
0.0	1.8	0
0.5	1.8	-12
1.0	1.8	-10
3.0	1.8	-12
5.0	1.8	-11
10	1.8	-13

Table 2. Calculated DNA concentrations from spectral data: GuHCl contamination. DNA concentrations are calculated using the A_{260} measurement and a extinction coefficient of 50 ng/µL/OD in the presence of the specified concentration of contaminant.

Phenol provides a second example of a commonly used reagent in molecular biology for the isolation of DNA, RNA and proteins. The method is commonly referred to as phenol-chloroform extraction and relies on a 1:1 ratio of water saturated phenol to chloroform. Precipitation of nucleic acids is generally performed subsequent to extraction for remove of residual phenol. It is obvious from figure 7 and table 3 that even trace amounts as low as 0.2% (v/v) of residual phenol can significantly impact accurate quantification of DNA.

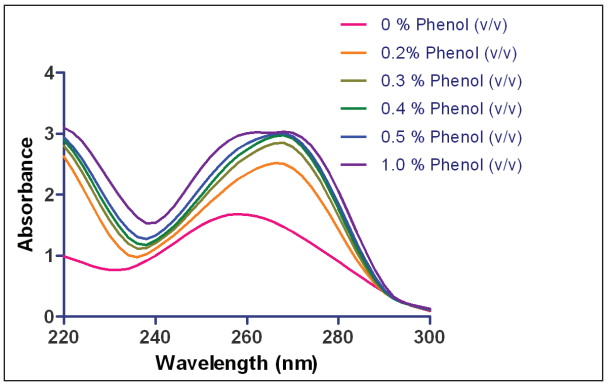

Figure 7. Spectral analysis of DNA: phenol contamination. Spectral analysis of herring sperm dsDNA spiked with increasing concentrations of phenol. Data normalized to a 1 cm path length.

[Phenol] ($\%^{v}/_{v}$)	A260/280	Calc'd [DNA] (% accuracy)
0.0	1.9	0
0.2	1.7	41
0.3	1.7	59
0.4	1.6	68
0.5	1.6	74
1.0	1.3	82

Table 3. Calculated DNA concentrations from spectral data: phenol contamination. DNA concentrations are calculated using the A_{260} measurement and an extinction coefficient of 50 ng/µL/OD in the presence of the specified concentration of contaminant.

Conclusion

The analysis highlights the ability to determine the concentration and purity of analyte in a sample volume as low as 2 µL using spectrophotometry. Additionally, examples of the ability to perform absorption spectral scans on low-volume samples are provided. The spectral data can be useful for detection of common residual contaminants from biomolecule isolation methods by comparison to spectra of highly purified sample. While the usefulness of the A_{260}/A_{280} ratio for determining the purity of the sample is understood, it is important to recognize the limitations of relying on the A_{260}/A_{280} ratio as a sole means for validation. Several supplementary methods exist to aid in validation of analyte purity including gel electrophoresis, mass spectroscopy and high-performance liquid chromatography (HPLC) that should be considered when accuracy is critical for downstream applications.

References

1. Brescia, P. and Banks, P. Multi-Volume Analysis of Nucleic Acids Using the Epoch/Take3 Spectrophotometer System, Winooski, (VT), BioTek Instruments, Inc., Oct. 2009, Application note.