Application Note: 50985

The Effect of Spectral Bandwidth on the Determination of Nucleic Acid Quantity and Purity

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Key Words

- DNA
- Protein
- RNA
- Semimicro Cells
- Spectral Bandwidth
- UV-Visible

Background and Introduction

In the modern biochemistry laboratory, the purity of DNA and RNA samples is frequently determined using the ratio of the solution UV absorption at 260 and 280 nm ($A_{260/280}$). A derivative of an assay originally developed by Warburg and Christian for the determination of nucleic acid impurity in protein samples,[1] the $A_{260/280}$ ratio is now relied upon to estimate the purity of nucleic acid samples.[2] Because the value of the ratio varies depending on the nucleic acid content of the DNA or RNA sample and the nature of the protein contaminant, this assay is frequently used to determine whether additional purification of the nucleic acid sample is necessary.

Differences in the absorption spectrum of DNA and proteins can be exploited to determine the purity of nucleic acids in solution. The UV absorption spectrum of all proteins has a maximum at 280 nm, primarily due to their aromatic amino acids. On the other hand, as shown in Figure 1, the sum of the individual absorption spectra of the four nucleic acid bases of DNA has a maximum near 260 nm. Intrinsically, a pure sample of DNA should have a $A_{260/280}$ between 1.7 and 2.0 depending on the individual nucleic acid sequence.[2] As the protein concentration increases, the $A_{260/280}$ ratio decreases, due to the increase in absorption at 280 nm.

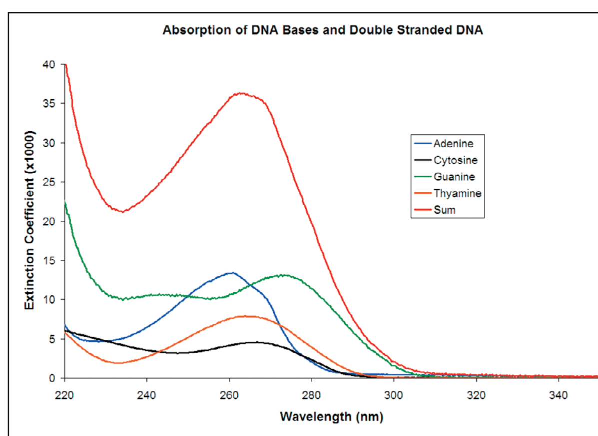

Figure 1: The UV absorption spectra of the individual DNA bases: adenine, cytosine, guanine, and thyamine. The sum of the four bases, representing the UV absorption of a fragment of DNA.

The $A_{260/280}$ assay is frequently employed because it is rapid and non-destructive. It relies only on the measurement of the absorption of the solution at two wavelengths and all of the solution used for analysis is preserved for future analysis use, resulting in essentially no loss of sample. Using 4.0 mm semimicro cells, this assay can be performed with as little as 450 µL of solution on spectrophotometers with a z axis of 8.5 mm. Our Evolution™ 300 and Evolution 600 UV-Visible spectrophotometers with Local Control have methods for these assays pre-programmed, making the assays even more simple and quicker to perform.

As shown in Figure 2, a method for determining the concentration and purity of nucleic acids using the $A_{260/280}$ ratio is pre-programmed in Local Control software. Also pre-programmed are assays measuring the $A_{260/280}$ and $A_{260/230}$ with scanning. This allows the user to visually inspect the purity of the sample and determine the wavelength of maximum DNA and protein absorption for more accurate ratios. With user-entered factors the DNA (260/280), DNA (260/230), DNA with Scan (260/280), DNA with Scan (260/230), dsDNA, ssDNA/RNA and oligos methods can determine the concentration of DNA(RNA) and protein in the sample. The Fixed application method files supplied with VISION*pro*™ software and the UVcalc spreadsheet functionality can be used to rapidly calculate the $A_{260/280}$ ratio.

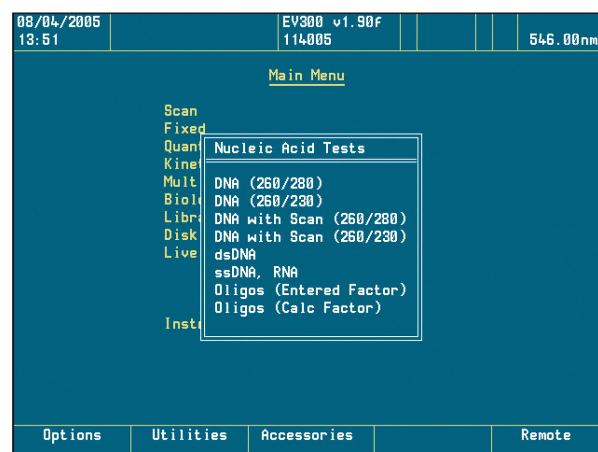

Figure 2: The Evolution 300/600 Local Control software displays the assays available for nucleic acid analysis

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The absorption spectrum of DNA is broad, with a full width at half maximum (FWHM) value of approximately 45 nm. Typically, a spectrophotometer with a spectral bandwidth (SBW) of one-tenth the FWHM of the band is required to resolve the peak. Thus, to resolve the maximum peak of the DNA spectrum, a SBW equal to or less than 4.5 nm is required. However, the measurements made for estimating the purity of DNA are most typically made by measuring the absorbance at fixed wavelengths. Therefore, the effect of SBW on the measurement is often ignored. To illustrate the effect of SBW on the measured absorbance value, the absorption spectrum of calf thymus DNA acquired at a SBW of 1.0 nm and 5.0 nm are shown in Figure 3.

In this application note, we examine the effect of spectral bandwidth on the determination of both the actual concentration of DNA from its absorption at 260 nm and the determination of the purity of the nucleic acid solution using the $A_{260/280}$ ratio.

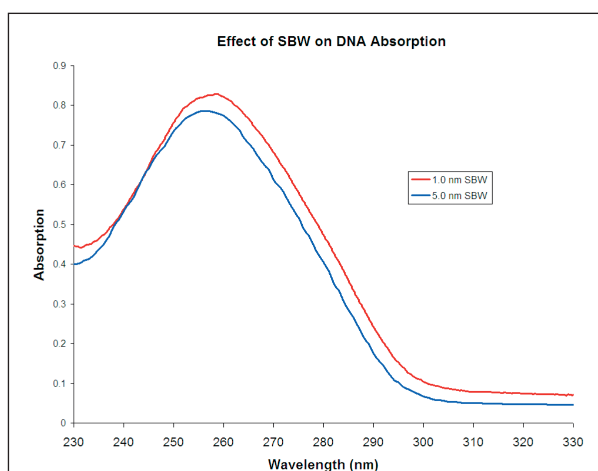

Figure 3: The absorption spectrum of calf thymus DNA acquired at 1.0 nm SBW and 5.0 nm SBW

Experimental

High molecular weight calf thymus DNA, purified by phenol/chloroform-extraction and lyophilized from DNase-free and RNase-free distilled deionized water, was used for the studies presented here. A stock solution was prepared by dissolving the lyophilized DNA into a saline sodium citrate (SSC) buffer (0.15 M NaCl, 0.015 sodium citrate, pH 7.0) at a concentration of approximately 2 mg/mL. The solution was stirred overnight to ensure complete dissolution. Serial dilutions of the stock solutions were prepared as presented in the results and discussion section below. The product information supplied with the lyophilized DNA indicated that the $A_{260/280}$ was determined to be 1.9, no information on the method used to make this determination was supplied in the specification.

An Evolution 300 UV-Visible spectrophotometer was used for the experiments presented here. The measurements were made using SBW's of 0.5, 1.0, 2.0 and 4.0 nm and an integration time of 2 seconds. A BioMate™ 3 spectrophotometer, with a SBW of 5.0 nm was also used for measurements presented here. All measurements were made in quartz 4.0 mm semimicro cells with an integration time of 2 seconds. Five replicates of the measurement were made and the average and standard deviation of the measurement were calculated either using the UVcalc spreadsheet functionality of the VISION*pro* software (Evolution 300 spectrophotometer) or by manual calculation (BioMate 3 spectrophotometer).

Table 1: Actual and Calculated Concentrations of DNA Solutions as a Function of SBW

Solution	Actual Concentration (ng/µL)	Calculated Concentration (ng/µL)				Deviation (ng/µL)				% Error			
		SBW (nm)				SBW (nm)				SBW (nm)			
		0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
1	199.75	202.93	201.96	203.66	203.32	3.18	2.21	3.91	3.57	1.6	1.1	1.9	1.8
2	99.88	95.98	95.14	95.24	94.98	3.90	4.74	4.63	4.90	3.9	4.9	4.9	5.1
3	49.94	47.64	47.01	46.58	46.11	2.30	2.93	3.36	3.83	4.6	6.2	7.2	8.2
4	9.99	8.87	8.88	8.69	8.61	1.12	1.11	1.30	1.38	11.2	12.5	14.6	15.9
5	5.00	4.43	4.44	4.33	4.29	0.56	0.56	0.67	0.71	11.3	12.6	15.1	16.4
6	2.50	2.11	2.17	2.07	2.02	0.39	0.33	0.43	0.48	15.4	15.6	20.1	23.4
7	1.25	1.06	1.11	0.97	0.98	0.19	0.14	0.28	0.27	15.4	13.0	25.4	27.5
8	0.63	0.50	0.51	0.50	0.46	0.12	0.12	0.13	0.17	19.2	23.2	25.4	33.5
		Total Deviation				**11.76**	**12.13**	**14.72**	**15.31**	**82.7**	**89.1**	**114.5**	**131.8**
		Average Deviation				**1.47**	**1.52**	**1.84**	**1.91**				

Table 2: $A_{260/280}$ Ratios as a Function of SBW

Solution	DNA Concentration (ng/µL)	$A_{260/280}$ Ratio				
		SBW (nm)				
		0.5	1.0	2.0	4.0	5.0
1	199.75	1.82	1.80	1.82	1.85	2.03
2	99.88	1.85	1.85	1.86	1.88	2.04
3	49.94	1.82	1.82	1.83	1.85	2.00
4	9.99	1.86	1.86	1.84	1.88	1.90
5	5.00	1.86	1.86	1.83	1.91	1.92
6	2.50	1.82	1.84	1.78	1.92	2.33
7	1.25	1.81	1.86	2.05	2.00	2.40
8	0.63	1.82	1.80	1.79	2.33	2.33
Average $A_{260/280}$ Ratio		**1.83**	**1.84**	**1.85**	**1.95**	**2.12**
Standard Deviation		**0.02**	**0.03**	**0.09**	**0.16**	**0.20**
Range		**0.05**	**0.06**	**0.28**	**0.48**	**0.50**

Results and Discussion

Quantity of DNA

Solutions ranging in concentration from approximately 200 to 0.6 ng/µL of calf thymus DNA were prepared by serial dilution from the stock solution described above. The results from the absorption measurements are shown in Table 1. The actual DNA concentrations reported in Table 1 were determined using an extinction coefficient of 0.0125 µL ng^{-1} cm^{-1}. This extinction coefficient was determined using a second independent set of five DNA solutions with concentrations ranging from 1.0 to 300 ng/µL (data not shown). For each SBW, a linear fit was constructed using the slope of the extinction coefficient given above and the appropriate y-intercept value for the data.

The second column section of Table 1 provides the deviation between the value calculated from the calibration curve at that SBW and the actual solution concentration. The third column section reports the percent error for the deviation. The total deviation and average deviation (the standard deviation) for each bandwidth is tabulated below the deviation columns. The total percent error is also tabulated from each bandwidth in the third column section.

As the solution concentration decreases, the calculated concentration of the DNA concentration consistently underestimates the actual amount of DNA present. As expected, the percent deviations increase monotonically as the actual concentration of DNA decreases. At a SBW of 0.5 nm, for solutions 50 ng/µL or higher in concentration, the deviation is less than 5%. The deviation increases to 11 – 15% for solutions between 10 and 1.25 ng/µL and is nearly 20% for solutions less than 1.0 ng/µL. However, at a SBW of 4.0 nm, the deviation increases to 8.2% above 50 ng/µL, 16 – 27.5% for solutions between 10 and 1.25 ng/µL, and over 30% for solutions less than 1.0 ng/µL, clearly indicating that the accuracy of the concentration measurement decreases as SBW increases.

The error in the DNA concentration results from the resolution of the absorption spectrum at the measurement wavelength. As mentioned previously, a general rule for the minimum resolution required to resolve the peak is one tenth of the FWHM of the peak. Here the influence of the width of the SBW is obvious. The increase in deviation between the actual solution concentration and the calculated concentration results from the increase in the amount of the absorption spectrum which is integrated. At a SBW of 0.5 nm, the approximate measurement region at 260 nm is 259.5 – 260.5 nm. However, at a SBW of 4.0 nm, the approximate measurement region at 260 nm is 256 – 264 nm. The overall increase in average deviation (standard deviation) of the measurements is a clear indication of the effect of increasing bandwidth on the overall quality of the measurement. Also evident from this data is the increase in the total deviation as a function of SBW.

Purity of DNA

The effect of SBW is also evident is in the determination of the nucleic acid purity from the $A_{260/280}$ ratio. The results from measurements on a pure DNA sample, described in the experimental section above, are given in Table 2. The $A_{260/280}$ ratio was determined on an Evolution 300 spectrophotometer with a SBW adjustable from 0.5 – 4.0 nm. The 5.0 nm SBW measurements were made on a BioMate 3 spectrophotometer.

As expected, the absorption data for each solution shows a decrease in the measured absorption as the SBW increases (see Figure 3). However, differences in the measured absorptions should be eliminated by the ratio measurement of the DNA purity. For example, at a SBW of 0.5 nm, the average $A_{260/280}$ ratio from the 8 solutions is 1.83 compared to a higher value of 2.12 determined with a SBW of 5.0 nm. The maximum variation observed in $A_{260/280}$ ratios obtained at SBWs of 0.5, 1.0, and 2.0 nm is 0.02, the variation in the ratio increases to a maximum of 0.27 at 5.0 nm.

The standard deviation and range observed in the $A_{260/280}$ ratio also increases as the SBW increases. This indicates that the accuracy and precision of the measurement of dilute DNA solutions begins to decline above a SBW of 2.0 nm. The dependence of concentration on the accuracy of the $A_{260/280}$ ratio is clear from Table 2. At concentrations below 2.5 ng/µL, the values obtained at a SBW greater than 1.0 nm show a slightly larger deviation. It is also important to note that because the variation in the observed $A_{260/280}$ ratio is nearly identical for a SBW of 0.5 – 2.0 nm, it is clear that the 4.0 mm width of the semimicro cell does not influence the quality of the measurements.

For measurements on very dilute samples or when elevated precision is necessary for comparing the purity of samples, the accuracy and precision of the $A_{260/280}$ ratio improves as the SBW decreases.

Conclusion

The data presented here clearly indicates the influence of the SBW on the accuracy of the determination of both the quantity and purity of nucleic acid samples. This application note shows that the quantity and purity of nucleic acid samples can be determined in quartz 4.0 semimicro cells, allowing the assay to be performed on sample volumes as low as 450 µL. It was observed that the deviation between the actual concentration and the calculated concentration of DNA increases as a function of SBW. While the purity measurements showed less variation than the quantization measurements, it is clear that when dilute solutions of nucleic acids are examined, a SBW less than or equal to 2.0 nm is preferred.

References

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