



Nucleic Acid Purity Assessment using A_{260}/A_{280} Ratios

A common practice in molecular biology is to perform a quick assessment of the purity of nucleic acid samples by determining the ratio of spectrophotometric absorbance of the sample at 260 nm to that of 280 nm. Here we describe the basis of A_{260}/A_{280} ratio determinations of nucleic acids and demonstrate the utility of the BioTek PowerWave™ 200 scanning microplate spectrophotometer to perform this procedure on large numbers of samples.

Introduction

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. Although this procedure was first described by Warburg and Christian (1) as a means to measure protein purity in the presence of nucleic acid contamination, it is most commonly used today to assess purity of nucleic acid samples. The basis of this test rests on the Beer-Lambert Law:

$$OD = eCb \quad (1)$$

Where the optical density (OD) is the product of the extinction coefficient (e), the concentration of the sample (C), and the optical pathlength (b). With an optical pathlength of 1 cm, which is commonly used in spectrophotometers, the pathlength can be ignored and extinction coefficients can be explained as an absorbance value at a specific concentration as seen in the equation below.

$$e = OD/C \quad (2)$$

The commonly accepted average extinction coefficients for 1 mg/ml nucleic acid solutions at 260 nm and 280 nm is 20 and 10 respectively. Similarly, the extinction coefficient values at 260 nm and 280 nm at a concentration of 1 mg/ml are 0.57 and 1.00 respectively for proteins. Thus in relative terms, nucleic acid samples would be expected to have a higher absorbance at 260 nm than at 280 nm, while with a protein sample, the inverse 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. The theoretical A_{260}/A_{280} ratio for samples that contain a mixture of protein and nucleic acid can be estimated by using the following formula:

$$A_{260}/A_{280} = \frac{(\epsilon_{260,p} \times (\%P) + \epsilon_{260,n} \times (\%N))}{(\epsilon_{280,p} \times (\%P) + \epsilon_{280,n} \times (\%N))} \quad (3)$$

Where %P and %N indicate the percentage of protein and nucleic acid respectively and the p and n subscripts refer to the extinction coefficients of protein and nucleic acid (2).

Materials and Methods

E. coli bacterial strain DH5a containing the plasmid pUC19 were grown in LB media at 37°C. Plasmid DNA was isolated and purified by alkaline lysis followed by cesium chloride gradient banding as described by Maniatis et. al. (3). Purified genomic herring sperm DNA was digested with Eco RI (Gibco-BRL, Gaithersburg, MD) followed by organic phenol/chloroform/isoamylalcohol (PCI) extraction and ethanol precipitation, with subsequent rehydration at

400 µg/ml final concentrations. Purified bovine serum albumin (BSA) fraction V, catalogue number A-2153 (Sigma, St. Louis, MO) was dissolved into distilled water at a concentration of 400 µg/ml and filter sterilized.

Spectrophotometric measurements were made using a Lambda 3B spectrophotometer (Perkin Elmer Corp., Norwalk, CT) with a band-pass setting of 1 nm. Samples were automatically blanked on water using a matched pair of Hellma quartz 1 cm cuvettes. Microplate measurements were made using a PowerWave 200 scanning microplate spectrophotometer (BioTek Instruments, Winooski, VT) in conjunction with Costar (Bedford, MA) UV transparent microplates, catalogue number 3635. Samples were blanked at each wavelength by pre-reading the plate at both wavelengths and subtracting the empty plate absorbance.

Results

The data depicted in Figure 1 demonstrates the positioning of peak absorbance of pure DNA and protein solutions, as well as a mixture of the two macromolecules. Each moiety demonstrates overlapping, but discernible peaks, with the peak in absorbance for DNA at 257 nm and for BSA protein at 277 nm. The protein sample also demonstrates a very high value below 240 nm that rapidly declines by 245 nm and most likely represents the absorbance of the peptide bonds in protein. A 10:1(w/w) mixture DNA:protein results in a peak absorbance of 259 nm and an absorbance profile very similar in shape as that demonstrated by pure DNA with a small increase at wavelengths below 240 nm and represents a sum of the two absorbance patterns of the macromolecules. The absorbance of all three samples falls to near zero above 300 nm (data not shown).

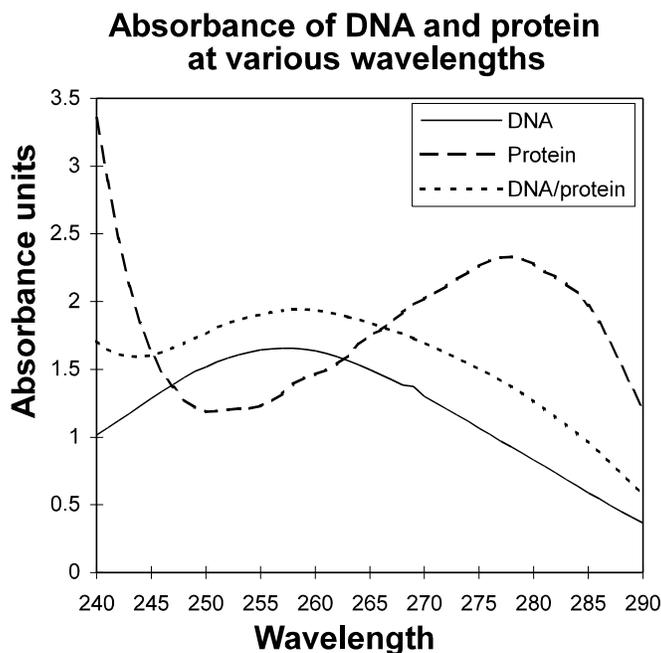


Figure 1. Absorbance profiles of DNA and protein samples from 240 to 290 nm. The absorbance of purified (¾¾) plasmid DNA (80 µg/ml); (-----) 3 mg/ml aqueous bovine serum albumin (BSA) solution; or a (- - - - -) 10:1 (w/w) DNA to protein mixture in aqueous solution was determined in 1 nm increments from 240 nm to 290 nm using a Perkin Elmer Lambda 3B spectrophotometer. Similar measurements were made using a 3 mg/ml aqueous bovine serum albumin (BSA) solution.

When the absorbances at different wavelengths are normalized by dividing by the value obtained at 280 nm an “A280 ratio” profile can be calculated. When this calculation is performed and plotted for pure DNA, a curve with a peak at 260 nm is generated (Figure 2). As would be

expected from the extinction coefficients for nucleic acids the value at this peak is 1.99, very close to the theoretically expected value of 2.0.

Likewise, samples containing only protein demonstrate a peak at 280 nm, reflecting the maximal absorbance of proteins at this wavelength. The sample containing a mixture of DNA and protein demonstrates a profile very similar to that of pure DNA in shape, but with values that are much lower, despite having equivalent amounts of nucleic acid in both samples.

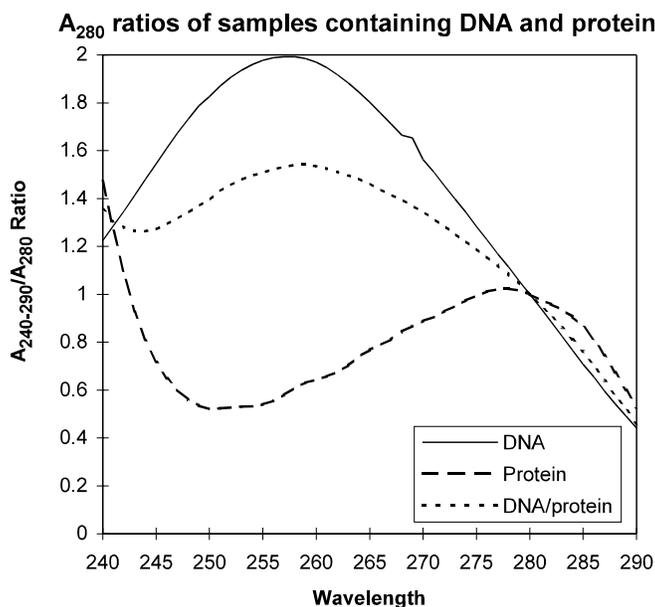


Figure 2. A₂₈₀ ratio of samples containing DNA and/or protein. Absorbance measurements were made on samples containing either DNA ($\frac{3}{4}$); or BSA protein (-----); or a mixture of both (- - - - -) at wavelengths from 240 nm to 290 nm. A₂₈₀ ratio measurements were then calculated by dividing the absorbance determination at each wavelength by the A₂₈₀ determination for that sample.

When the A₂₆₀/A₂₈₀ ratio is determined for a range of different DNA/protein mixtures one finds that the ratio is relatively insensitive to the addition of protein to pure nucleic acid. As demonstrated in Figure 3 as increasing percentages of protein are measured little change is seen in the A₂₆₀/A₂₈₀ ratio until the percentage of protein is approximately 75%. Interestingly, even when equal amounts of nucleic acid and protein by weight are determined a ratio of 1.75 is still returned. DNA or protein only samples were found to have A₂₆₀/A₂₈₀ ratios of 1.92 and 0.64 respectively.

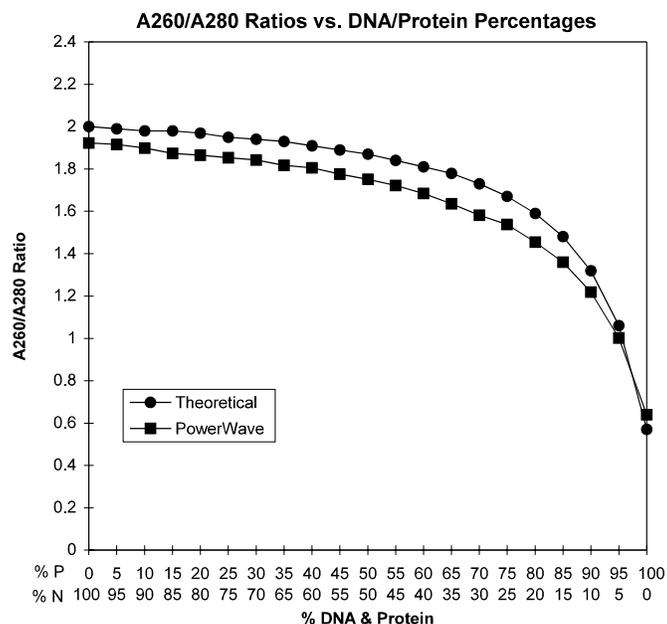


Figure 3. Comparison of theoretical A260/A280 ratios with those determined using the PowerWave 200 scanning microplate spectrophotometer. The absorbance of various mixtures of DNA and protein were determined at 260 nm and 280 nm using a BioTek Instruments PowerWave 200 scanning microplate reader. Subsequently the A260/A280 ratios were determined for each mixture and compared to the theoretical value calculated from the extinction coefficients. Filled circles (●) indicates theoretical ratios calculated using equation 3, while filled boxed (■) denote experimentally determined ratios.

Discussion

It is important to note that the A260/A280 ratio is only an indication of purity (2, 3) rather than a precise answer. Pure DNA and RNA preparations have expected A260/A280 ratios of ³1.8 and ³2.0 respectively (3) and are based on the extinction coefficients of nucleic acids at 260 nm and 280 nm. Although the A260/A280 ratio is relatively insensitive to change and seemingly useless when DNA/protein mixtures are experimentally tested, the utility of this procedure becomes apparent when nucleic acids are purified from tissue or blood. Tissue samples and to a lesser extent whole cells have a protein content that greatly exceeds that of nucleic acid on a weight basis and purification of samples to a A260/A280 ratio represents an enrichment of nucleic acid that could be as much as 1 million fold.

Several factors may affect A260/A280 ratios. The 260 nm measurements are made very near the peak of the absorbance spectrum for nucleic acids, while the 280 nm measurement is located in a portion of the spectrum that has a very steep slope. As a result, very small differences in the wavelength in and around 280 nm will effect greater changes in the A260/A280 ratio than small differences at 260 nm. Consequently, different instruments will result in slightly different A260/A280 ratios on the same solution due to the variability of wavelength accuracy between instruments. Individual instruments, however, should give consistent results. Concentration can also affect the results, as dilute samples will have very little difference between the absorbance at 260 nm and that at 280 nm. With very small differences, the detection limit and resolution of the instrument measurements begin to become much more significant. The type(s) of protein present in a mixture of DNA and protein can also affect the A260/A280 ratio determination. Absorbance in the UV range of proteins is primarily the result of aromatic ring structures. Proteins are composed of 22 different amino acids of which only three contain aromatic side chains. Thus, the amino acid sequence of proteins would be expected to have a tremendous influence on the ability of a protein to absorb light at 280 nm. A protein with a very high content of amino acids with aromatic side chains would in turn have a higher extinction coefficient than a protein with very few. For example, BSA has an extinction

coefficient value of 0.7 for a 1 mg/ml solution at 280 nm, while streptavidin, on the other hand, with an extinction coefficient of 3.4 absorbs almost five times as much light at 280 nm at the same concentration.

In the past, such A260/A280 ratio determinations have been performed using the conventional spectrophotometer. This method usually entailed using a pair or at most a set of four matched cuvettes to perform the analysis resulting in a very low throughput. The ability to use the PowerWave 200 scanning microplate reader to perform this analysis allows this routine procedure to be performed on 96 samples in a matter of seconds leading to a tremendous increase in productivity and throughput.

References

1. Warburg, O. and W. Christian (1942) Isolation and crystallization of enolase. *Biochem. Z.* 310:384-421.
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3. Maniatis T., E.F. Fritsch, and J. Sambrook (1982) *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Springs Harbor, NY.

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