Measure Your Purity

Assessment of Nucleic Acid Purity *via* UV Absorbance

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Molecular biologists commonly require assessment of nucleic acid sample purity through a spectrophotometric absorbance ratio of the sample at 260 nm compared to that at 280 nm. Impurities common to nucleic acid preparations that cannot be detected at these wavelengths can often be detected at 240 nm. The basis of nucleic acid A_{260}/A_{280} ratio determinations and the use of 240 nm measurements to detect common contaminants are described below.

Purification of intact nucleic acid from samples is required for many molecular biology applications. Accurate PCR replication of RNA and DNA sequences requires complete removal of cellular lipids and proteins. Likewise, most restriction endonucleases used to digest genomic DNA are inactivated or degraded by cellular proteases normally present prior to purification of the nucleic acids. Thus, failure to remove these cellular contaminants often leads to poor results.

The assessment of the purity of a nucleic acid sample is usually performed by a procedure known as the A_{260}/A_{280} or 260/280 ratio. While this procedure was first described by Warburg and Christian in 1942 to assess protein purity in the presence of nucleic acid contamination [1] it is now the most common method used to measure nucleic acid purity and is supported by the Beer-Lambert Law:

Eq. 1 $\mathbf{OD} = \varepsilon \boldsymbol{C} \boldsymbol{b}$

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

Eq. 2 $\varepsilon = \mathbf{OD}/\boldsymbol{C}$

The commonly accepted average extinction coefficients for nucleic acid solutions at 260 nm and 280 nm are 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 are expected to have a higher absorbance at 260 nm than at 280 nm, while the inverse would be true for a protein sample. Using these extinction coefficients, pure nucleic acid samples would have an A_{260}/A_{280} ratio approaching 2.0, while protein would have a ratio approaching 0.57. Samples containing a protein and DNA mixture would, of course, be influenced by both macromolecules. The theoretical A_{260}/A_{280} ratio for samples with a mixture of protein and nucleic acid can be estimated by using the following formula:

Eq. 3
$$\mathbf{A_{260}/A_{280}} = \frac{(\varepsilon_{260,p} \times (\%P) + \varepsilon_{260,n} \times (\%N))}{(\varepsilon_{280,p} \times (\%P) + \varepsilon_{280,n} \times (\%N))}$$

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].

Unfortunately, the A_{260}/A_{280} ratio is primarily intended to assess protein contamination. Many compounds, including chemicals commonly employed in nucleic acid preparations, absorb light at wavelengths below 260 nm. While these compounds are useful during purification, they may denature proteins or inhibit reactions in later steps, thus negatively affecting or destroying the experiments. For many of these contaminating compounds, measurement at 240 nm and a subsequent A_{260}/A_{240} ratio can provide insight into the extent of other contamination.

Materials and Methods

Molecular biology grade reagents including sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and phenol were purchased from Invitrogen (Carlsbad, CA). Solutions including 10 % SDS, 0.5 M EDTA and phenol were prepared as described by Maniatis *et al.* [3] *E. coli* DH5α 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 (Invitrogen) followed by organic phenol/chloroform/isoamylalcohol (PCI) extraction and ethanol precipitation, with subsequent rehydration at 400-µg/mL final concentration. Purified bovine serum albumin (BSA) fraction V (Sigma, St. Louis, MO) was dissolved into distilled water at a concentration of 400 µg/mL and filter sterilised.

A series of stock solutions were made by mixing differing amounts of the 400 µg/mL DNA and protein stock solutions. In each of these DNA/protein mixtures, 1 % by volume of SDS, phenol/chloroform/isoamylalcohol (PCI) or EDTA was added to a final concentration of 1 % PCI, 0.1 % SDS, and 5mM EDTA.

Spectrophotometric measurements were made either using a cuvette-based Lambda 3B spectrophotometer (PerkinElmer Corp., Norwalk, CT) or a PowerWave microplate spectrophotometer (BioTek Instruments, Winooski, VT). Spectrophotometric measurements were made using a matched pair of Hellma quartz 1 cm cuvettes and automatically blanked on water. Microplate measurements were made in UV transparent microplates (Corning, Corning, NY) and blanked at each wavelength by subtracting from each well the average absorbance of 12 wells containing only water. Raw data was exported to a Microsoft Excel spreadsheet and plotted using GraphPad 4 software.

Results

Peak absorbances of pure DNA and protein solutions are clearly seen in figure 1, as well as a peak for the mixture of the two macromolecules. Each moiety demonstrates overlapping but discernible peaks, with a peak in absorbance for DNA at 257 nm and for BSA protein at 277 nm. A 10:1($^{w}/_{w}$) DNA to protein mixture results in a peak absorbance of 259 nm and an absorbance profile very similar to that demonstrated by pure DNA with a small increase at wavelengths below 240 nm. This represents a sum of the absorbance patterns of both macromolecules.

Absorbance values at different wavelengths can be normalised by dividing by a 280 nm value to calculate an A_{280} ratio profile. When this calculation is plotted for pure DNA, a curve with a peak

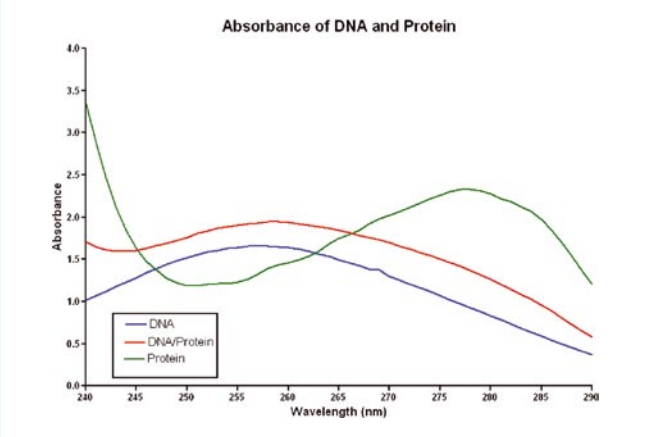

Fig. 1: The absorbance of purified plasma DNA (80 µg/mL); 3 mg/mL aqueous BSA solution; or a 10:1 (w/w) DNA to protein mixture in aqueous solution was measured in 1 nm increments from 240 nm to 290 nm using a traditional spectrophotometer.

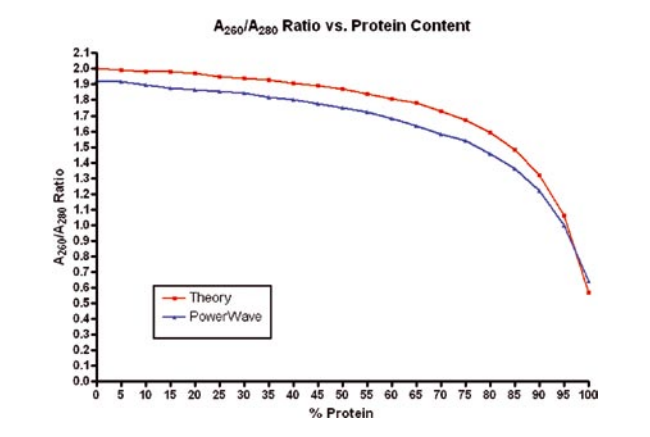

Fig. 3: Comparison of theoretical A_{260}/A_{280} ratios with those determined using a microplate spectrophotometer. The absorbances of various DNA/protein mixtures were determined at 260 nm and 280 nm. A_{260}/A_{280} ratios were calculated for each mixture and compared to the theoretical value from the extinction coefficients.

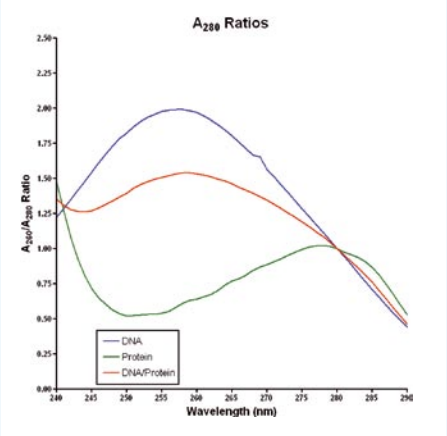

Fig. 2: A_{280} ratio measurements were calculated for DNA, BSA protein and DNA/protein samples by dividing the absorbance determination at each wavelength by the A_{280} determination for that sample.

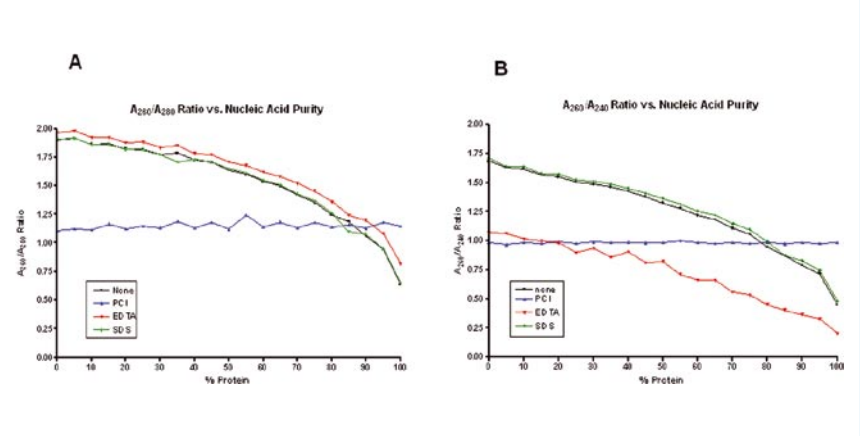

Fig. 4: Comparison of A_{260}/A_{280} and A_{260}/A_{240} ratios of DNA/protein mixtures containing trace amounts of common contaminants of nucleic acid preparations. Values represent a mean of eight determinations.

at 260 nm is generated (fig. 2). As would be expected from the extinction coefficients for nucleic acids, the value at this peak is 1.99, approaching the 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, but with values that are much lower, despite having equivalent amounts of nucleic acid in both samples.

When the A_{260}/A_{280} ratio is determined for a range of different DNA/protein mixtures, one finds that the ratio is relatively unaffected by the addition of protein to pure nucleic acid. As increasing percentages of protein are measured (fig. 3), very little change is seen in the A_{260}/A_{280} 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 A_{260}/A_{280} ratios of 1.92 and 0.64 respectively.

The absorbance for a series of DNA/protein mixtures were measured at 240 nm, 260 nm, and 280 nm, and A_{260}/A_{280} and A_{260}/A_{240} ratios were calculated. As demonstrated in figure 4, DNA/protein mixtures with trace amounts of SDS or EDTA cannot be distinguished from mixtures without these contaminants when A_{260}/A_{280} ratios are determined. When phenol is present, however, a dramatic change is seen in the A_{260}/A_{280} ratio.

When the absorbance at 240 nm of these samples is determined, EDTA contamination of nucleic acid samples can be distinguished. Therefore, the ratio of absorbance at 260 nm to that of 240 nm can easily determine the presence of EDTA. The presence of PCI can also be distinguished at 240 nm and results in an A_{260}/A_{240} ratio similar to that seen with the A_{260}/A_{280} ratio. SDS contamination cannot be detected using this ratio.

Discussion

It is important to note that the A_{260}/A_{280} ratio is only an indication of purity [2–3] rather than a precise answer. Pure DNA and RNA preparations have expected A_{260}/A_{280} ratios of ≥1.8 and ≥ 2.0 respectively [3] and are based on the extinction coefficients of nucleic acids at 260 nm and 280 nm. Although the A_{260}/A_{280} ratio is relatively insensitive to change and seemingly ineffective 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 A_{260}/A_{280}

ratio represents an enrichment of nucleic acid that could be as high as 1 million fold.

Several factors may influence A_{260}/A_{280} ratios. Measurements at 260 nm are near the nucleic acid absorbance spectrum peak, while those at 280 nm are located in a portion of the spectrum with a steep slope. As a result, very small differences in the wavelength in and around 280 nm will produce greater changes in the A_{260}/A_{280} ratio than small differences at 260 nm. Consequently, different absorbance instruments will result in slightly different A_{260}/A_{280} ratios on the same solution due to the variability of wavelength accuracy between instruments. Properly maintained instruments, however, should provide reproducible and consistent results.

Sample 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 increase in significance. This is exacerbated in microplate-based measurements, where the pathlength is usually less than that of standard cuvettes.

The type(s) of protein present in a DNA/protein mixture can also affect the A_{260}/A_{280} ratio determination. Protein absorbance in the UV range is primarily the result of aromatic ring structures found in certain amino acids that make up the protein. 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 amino acid aromatic side chains. For example, BSA has an extinction coefficient value of 0.7 for a 1 mg/mL solution at 280 nm, while streptavidin with an extinction coefficient of 3.4 absorbs almost five times as much light at 280 nm at the same concentration.

There are several differences between spectrophotometers and microplate readers that should be addressed. Typically, spectrophotometers use matched quartz cuvettes with relatively consistent background absorbance for UV measurements, automatically subtracting buffer and vessel backgrounds at each wavelength. For microplate-based applications, quartz microplates are extremely expensive, and most researchers choose instead to use disposable UV transparent microplates. Although more readily available and convenient, disposable plastics have a less consistent background. In order to compensate for any variability, blanking with one or more wells to remove vessel background should be performed for each wavelength used.

Unlike spectrophotometers, the pathlength of the absorbing solution in microplates is not fixed at 1 cm, but is dependent upon several parameters, the most influential of which is the volume of solution in each well. The depth of the solution (i.e., pathlength) is directly proportional to the volume of solution in the well. For ratiometric analysis (e.g. A_{260}/A_{280} or A_{260}/A_{240}) this is generally not a concern, however, a direct nucleic acid quantitation calculation based on the A_{260} value is often made with the same measurement. For this calculation a correction to a 1 cm value is necessary [4].

References

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