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Application Note

Nucleic Acid Quantitation Using BioTek's Scanning Microplate Spectrophotometer

Abstract

An essential element of cellular and molecular biology is the ability to quantitate nucleic acids in large numbers of samples at a sensitivity that enables determination of small amounts of sample. Here we describe methodology to quantitate nucleic acids using the BioTek PowerWave™ HT Scanning Microplate Spectrophotometer.

Introduction

Research investigators from many different fields of study are required to quantitate various nucleic acids in large numbers of samples. Southern blot analysis requires that equivalent levels of genomic DNA be loaded in each well. Likewise, Northern blot analysis requires equal amounts of total or poly A^+ RNA be run in each well if comparisons are being made. Quantitation of PCR products or plasmid minipreps prior to DNA sequencing has been found to be a critical step in successful DNA sequencing. Quantitation of synthesized oligonucleotides is routinely used to optimize PCR or primer extension experiments. The common element to all of these diverse molecular biological procedures is the necessity to determine the concentration of a nucleic acid.

Although there have been a number of different methodologies developed to quantitate nucleic acids, absorbance at 260 nm remains the de facto standard by which all other methodologies are measured. Early methods utilized measurements of hydrolyzed nucleic acids and by the quantitation of the various components from hydrolysis, such as the phosphorous or the ribose and/or deoxyribose sugar of the backbone, make estimates of total nucleic acid concentrations (1, 2). These assays are time consuming and relatively insensitive. DNA specific assays for hydrolyzed samples (3, 4) with improved sensitivity have also been developed and adapted for cellular DNA synthetic rates (5). These assays all suffer because they inherently destroy the nucleic acid sample. Recent advances with intercalating fluorescent dyes such as ethidium bromide (6); Hoechst dye 33258 (7); and PicoGreen® (8), have improved sensitivity to the picogram level, but are relatively specific for dsDNA limiting their utility in the determination of RNA or oligonucleotide concentrations. The direct quantitation of nucleic acids using the spectrophotometric absorbance at 260 nm in conjunction with the appropriate extinction coefficients (9) remains the easiest and most versatile method to quantitate nucleic acids in aqueous solution.

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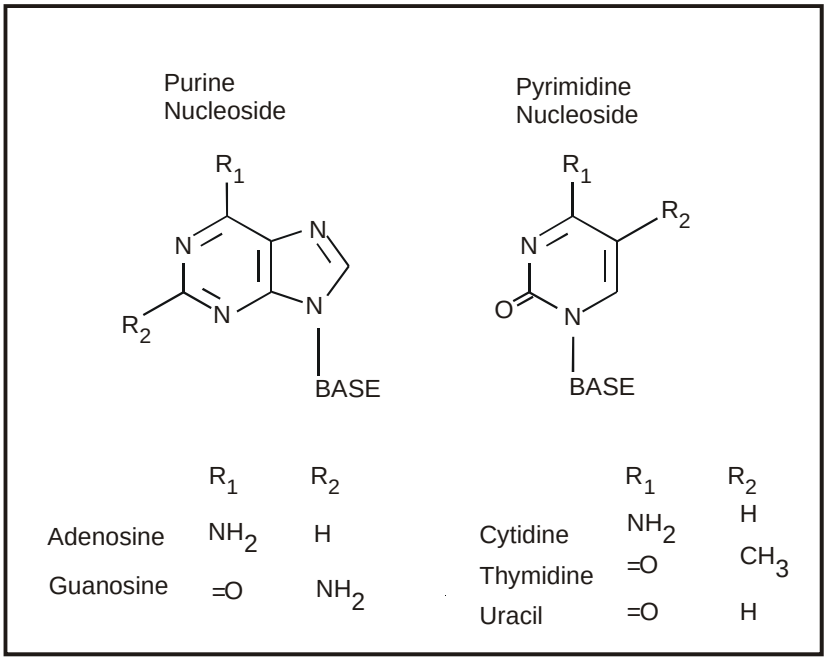

Figure 1. Aromatic purine and pyrimidine ring structure of nucleoside bases. Differing side groups for all five different nucleosides are indicated by R_1 and R_2.

The aromatic ring structure of the purine and pyrimidine moieties that make up the nucleoside bases of DNA and RNA are responsible for absorbance of UV light at 260 nm (Figure 1). Although each specific base has a maximal absorbance at a slightly different wavelength, on average, nucleic acids as a macromolecule will absorb maximally very near 260 nm. The direct conversion from absorbance to concentration requires that the pathlength of the absorbing material be known. Horizontal photometry, as performed in spectrophotometers, utilizes physical dimensions of the cuvette to fix the light pathlength of the sample. This light pathlength has been standardized to 1 cm in order to allow easy comparison of data. In vertical photometry, as performed by microplate readers, the light passes through the vertical axis of the absorbing solution. This results in a situation where the pathlength of the absorbing solution is dependent on the volume of the solution. In order for vertical photometric devices to be able to directly quantitate solutions, absorbance measurements need to be corrected to 1 cm. The pathlength of any dye solution in a microplate well can be determined by comparing the absorbance of that dye solution at a known pathlength (e.g. 1 cm) to the absorbance in the microplate well [10]. The ratio of the microplate well absorbance determination to the 1 cm determination is equal to the pathlength in centimeters. Much like a dye solution, water has a small yet significant peak in absorbance at 977 nm [11]. BioTek takes advantage of this and the fact that DNA samples are generally in an aqueous solution.

Water has virtually no absorbance between 200 and 900 nm, but it has a small but significant peak at 977 nm [11]. The microplate reader determines the pathlength of the sample by measuring the absorbance of the water in the microplate sample at 977 nm, blanks that value using a reference wavelength of 900 and compares it to the absorbance of water at 1 cm. The ratio of those two values will be the pathlength of the sample (Eq. 1).

$$\frac{(A_{977}-A_{900})_{sample}}{(A_{977}-A_{900})_{1.0\ cm\ water}} = \text{Pathlength of sample}$$

Eq.1

Water has an absorbance at 977 nm of 0.18 with a 1-cm pathlength. This value, often referred to as a "k" value, is a constant and can be used as such to replace the denominator in equation 1. Other solvents would be expected to require a different pathlength correction wavelength and "k" factor.

As background absorption of the microplate is generally quite high in the UV range (below 340 nm), it is particularly important with UV absorbance measurements to blank prior to pathlength correction in order to obtain an accurate corrected value. Correction to 1 cm for DNA samples, which is determined using the absorbance at 260 nm, would need to accommodate this (Eq. 2).

$$\frac{((A260_{sample})-(A260_{blank}))}{\text{Pathlength of sample}} = \text{Absorbance of sample corrected to 1.0 cm}$$

Eq. 2

These calculations are automatically performed if pathlength correction has been enabled via software commands (Figure 4). With pathlength correction, the reader automatically measures the absorbance of each well at the selected pathlength correction wavelengths and calculates the pathlength of each sample using the supplied "k" value. Note that the default values for KC4, which are a pathlength wavelength of 977 nm, a reference wavelength of 900 nm and a "k" value of 0.18, have been optimized for aqueous samples.

In terms of quantitation, different nucleic acid species have different average extinction coefficients. By converting these known extinction coefficients to a specific concentration for a particular absorbance value at a defined wavelength, the nucleic acid concentration of solutions can be determined by a simple absorbance measurement. For example, the extinction coefficient for dsDNA (1 mg/ml) at 260 nm is 20 OD units; this can be rearranged to mean that a DNA sample that returns an absorbance 1.0 OD unit has a concentration of 50 µg/ml. Similar calculations have been performed for ssDNA (oligonucleotides) and RNA species (see Table 1).

Nucleic Acid Species	Concentration for 1 A260 Unit (µg/ml)
dsDNA	50
ssDNA (oligonucleotides)	33
ssRNA	40

Table 1. Commonly accepted absorbance to concentration conversion for nucleic acids. Indicated values are accepted average values for absorbance measurements at 260 nm with a 1 cm pathlength.

Materials and Methods

The 96-well clear UV transparent microplates (catalog number 3635) were purchased from Costar (Cambridge, MA). Purified genomic herring sperm DNA (catalog number D-1816) was purchased from Promega (Madison, WI) and Tris and EDTA were obtained from Sigma-Aldrich (St Louis, MO). Several series of dilutions of purified herring sperm DNA were made using TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) as the diluent. Following dilution, 100-, 200- and 300-µl aliquots were pipetted into microplate wells and absorbance measurements were then made using a PowerWave™ HT Scanning Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). The background absorbance of the microplate was first subtracted using the pre-read feature of the KC4™ software package (BioTek Instruments). Samples were also blanked using the mean absorbance of TE buffer blanks in the same microplate. Determination of the concentration for unknown samples in microplates was carried out by measuring absorbance at 260 nm followed by correction for light pathlength. The subsequent pathlength corrected absorbance was then transformed using the appropriate conversion factor indicated in Table 1.

Results

The absorbance was determined at 260 nm using the PowerWave™ HT Scanning Microplate Spectrophotometer for DNA concentrations ranging from 0 to 100 µg/ml at three different volumes. Over this concentration range the intensity increased in a linear fashion (Figure 2). As predicted by the Beer-Lambert equation, samples with a greater volume consistently returned a higher absorbance value than those with lower volumes despite having the same expected DNA concentration. Using Microsoft® Excel™, a least means squared linear regression analysis was generated with a coefficient of determination (r^2) value of 0.999 (Figure 2). The average coefficient of variance (%CV) of the standards was less than 3%, with the greatest percent variation taking place in the lower DNA concentrations tested (data not shown). In terms of sensitivity, this assay method was found sensitive to a DNA concentration of approximately 390 ng/ml or roughly a 100-ng/well minimum assay concentration.

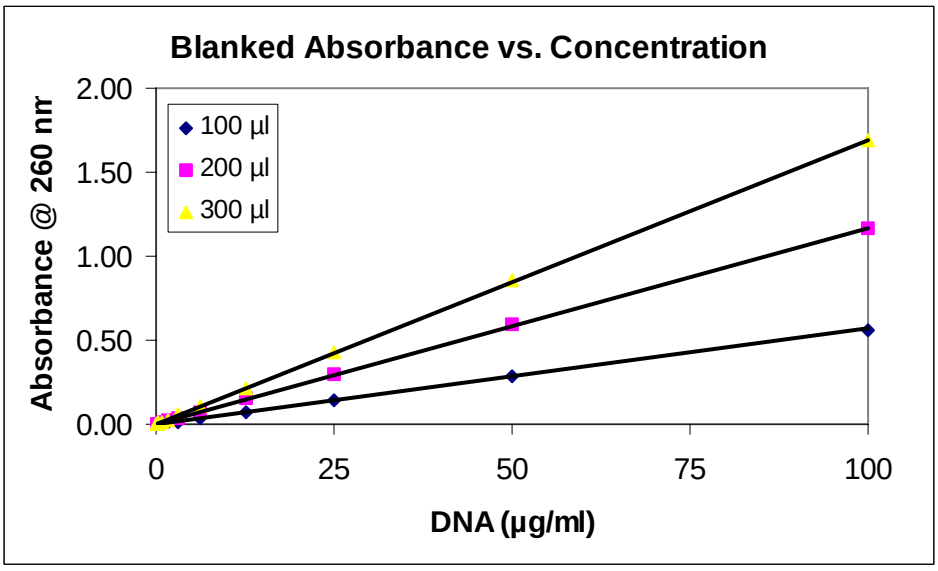

Figure 2. Comparison of absorbance values from different sample volumes. The absorbance at 260 nm of the same DNA samples at different well volumes were determined using the PowerWave™ HT and compared. Solid lines represent linear regression analysis of the data, while the markers represent the mean of eight determinations.

When pathlength correction is enabled with the same samples, a markedly different result is returned. All three volumes result in virtually the same corrected absorbance (Figure 3). In these experiments, differences in the absorbance of the samples at 977 nm serve to normalize the data. When the corresponding transformation that converts absorbance at 260 nm to DNA concentration is employed, the different volumes return similar concentrations, despite the very different raw absorbance values. This feature can also be utilized to correct for pipetting volume errors. Wells receiving less than the expected volume of fluid would normally return an experimental absorbance value less than the corresponding well with the correct volume of fluid. Pathlength correction normalizes all the wells to a common pathlength, which is directly related to volume. This is demonstrated in Figure 3, where samples with the same DNA concentration but different sample volumes, result in very similar corrected 260-nm absorbance values. Because background absorbance of the microplate can be significant in the UV region, particularly with very low DNA concentration, plates were pre-read empty prior to adding DNA samples (Figure 4). This feature allows the background absorbance for each well to be individually subtracted rather than using one or two wells to represent the remainder. A buffer blank was also defined to subtract any background absorbance resulting from the TE buffer diluent.

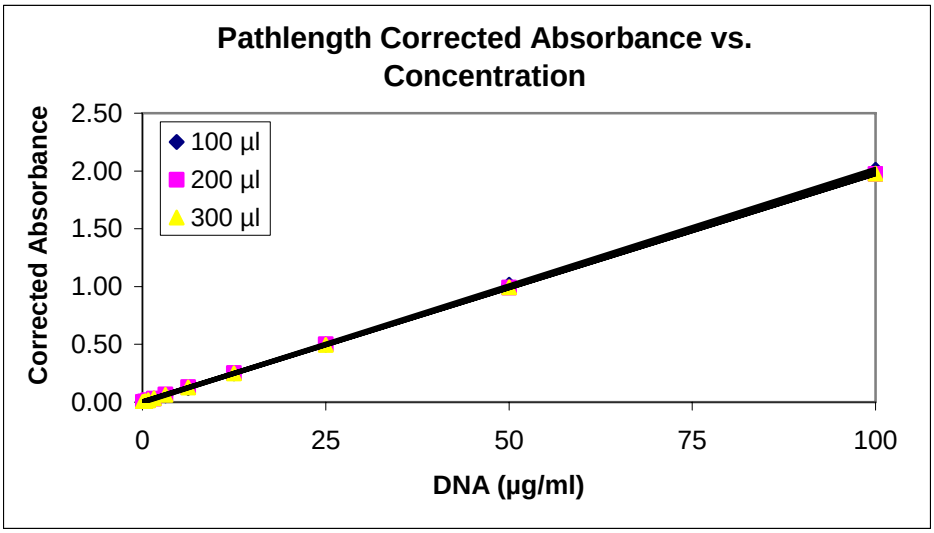

Figure 3. Pathlength Corrected Absorbance Values. The automatic pathlength correction feature of KC4™, the pathlength each well was determined using the absorbance of water in the aqueous samples and comparing it to a known standard. The experimental values were then corrected to 1 cm and plotted using Microsoft® Excel™.

One of the primary concerns with microplate determinations is the affect of the air-liquid interface on pathlength correction (Figure 4). When the DNA concentrations of samples in microplates are determined with the PowerWave™ HT the results compare very well to those determined directly using a Bio-Cell™ (Table 2). The Bio-Cell allows for true 1-cm fixed pathlength absorbance determinations to be made in microplate readers. The patented design has two parallel optical surfaces 1 cm apart in the vertical axis, eliminating the air liquid interface meniscus.

Expected DNA Conc. (µg/ml)	PowerWave HT 100 µl*	200 µl*	300 µl*	Bio-Cell
100	100.51	98.71	98.79	101.20
50	50.79	49.52	49.64	51.10
25	24.98	25.02	24.82	25.75
12.5	12.47	12.63	12.46	13.00
6.25	6.16	6.47	6.34	6.50
3.13	2.93	3.32	3.16	3.25
1.56	1.57	1.52	1.70	1.60
0.78	0.63	0.77	0.84	0.80
0.39	0.20	0.34	0.35	0.55
0.20	0.15	0.14	0.185	0.30
0.00	-0.13	-0.01	-0.04	0.00

- Mean of eight determinations

Table 2. Comparison of DNA concentrations determined by the Use of Microplates or a Bio-Cell™. Concentration determinations were made by measuring the absorbance at 260 nm with a PowerWave™ HT using Costar 3635 microplates or a Bio-Cell. Determinations were made using 100 µl, 200 µl, and 300 µl sample volumes in each well. In order to determine concentration values, the absorbance of samples measured with the PowerWave™ HT were corrected for light path length prior to transformation to a concentration. The PowerWave results represent the mean of eight determinations, while the Bio-Cell data represents the mean of duplicate determinations.

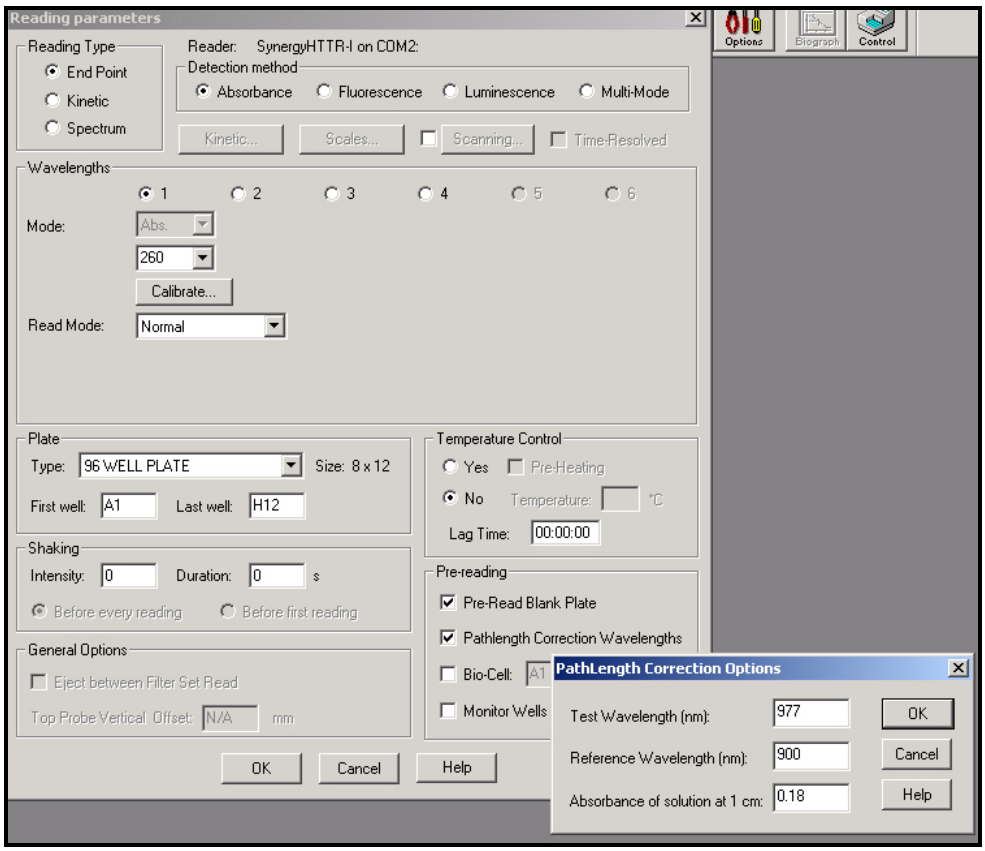

Figure 4. KC4 Protocol Reading Parameters Screen Shot. Reading parameters for nucleic quantitation utilize an absorbance measurement at 260 nm with pathlength correction prior to transformation from absorbance to concentration. Pre-read blank plate is selected in order to subtract the background absorbance of the microplate. Note the pathlength correction options box indicating the default values for water including a test wavelength of 977 nm and a 1-cm solution absorbance of 0.18.

Discussion

Although there are several methods that are commonly used to determine nucleic acid concentration, the most commonly employed method involves the spectrophotometric determination of nucleic acid concentration by absorption at 260 nm. Because all of the bases of nucleic acid have either a purine or a pyrimidine ring structure, absorbance at 260 nm can be utilized to determine concentrations of nucleic acids with a high degree of confidence from virtually all sources. Proteins, on the other hand, contain variable amounts of UV absorbing aromatic amino acids, which makes the direct conversion of absorbance to concentration difficult, particularly when purified or semi-purified proteins are being quantitated.

Unlike spectrophotometers, the pathlength of the absorbing solution in microplates is not fixed at 1 cm, but is dependent upon several parameters that can affect the depth of the solution in the well. The most influential parameter to affect the light pathlength is the volume of solution added to each well for an absorbance determination. The depth of the solution (i.e., pathlength) is directly proportional to the volume of solution in the well. In flat bottom microplate wells the pathlength for 200 µl of a DNA solution in a well would be expected to be twice that of 100 µl. The shape of the microplate well will also influence the pathlength. Round bottom wells, because of their shape, will have a greater light pathlength than a flat-bottomed well for the same volume of fluid. Similarly, half-volume microplates would be expected to result in a greater pathlength than normal microplate wells for a given volume of solution due to their reduced size. The pathlength is also dependent on the meniscus of the solution. The presence of detergents or surfactants will reduce the surface tension and result in a concave shaped meniscus and effectively reduce the light pathlength of a sample in a well.

Although in this report only dsDNA in aqueous solution was tested, other nucleic acids can be quantitated. By using the absorbance to concentration conversions outlined in Table 1 RNA samples or ssDNA (oligonucleotides or M13 phage DNA) can be quantified in microplates. As described for DNA, the absorbance at 260 nm needs to be corrected for path length in order for the direct conversion from absorbance to concentration to return valid results.

Accurate nucleic acid determinations require that absorbance measurements be made in the linear range of the reader. At high DNA concentrations the absorbance at 260 nm can be beyond the linear range of the reader. At these levels the increase in absorbance as measured by the reader is no longer proportional to the increase in DNA concentrations. Subsequent transformation to concentration would result in a calculated concentration lower than what is actually present. At very low absorbance values, the error of measurement becomes a significant proportion of the determinations. While one can subtract well-to-well differences in background absorbance caused by the microplate, small read-to-read variations and meniscus variability can result in variations of 0.001 to 0.002 ODs. At very low nucleic acid concentrations this can be a significant percentage of the measurement.

In the past such A_{260} determinations have been performed using a 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 perform DNA quantitation in microplates allows this routine procedure to be performed on 96 samples in a matter of seconds leading to a tremendous increase in productivity and throughput. While this application note used the PowerWave™ HT to perform this analysis, any one of the microplate readers with absorbance scanning capabilities (e.g. the Synergy™ HT, PowerWave™ XS, and µQuant™) are capable of these measurements.

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