

Calculating Nucleic Acid or Protein Concentration

Using the GloMax[®]-Multi+ Detection System

INTRODUCTION

Direct absorbance (also called optical density or OD) measurements of nucleic acid samples at 260nm (OD₂₆₀) or protein samples at 280nm (OD₂₈₀) can be converted to concentration using the Beer-Lambert law, which relates absorbance to concentration using the pathlength of the measurement and an extinction coefficient [1].

$$\text{Beer-Lambert Equation} \quad A = \epsilon c l$$

Where A = absorbance, ϵ = molar extinction coefficient, c = concentration (in the units corresponding to ϵ) and l = light pathlength. Given this equation, concentration can be calculated by:

$$\text{Concentration} = \frac{A}{\epsilon l}$$

EXTINCTION COEFFICIENTS

Extinction coefficients have been calculated for specific nucleotide groups (Table 1). Using the Beer - Lambert equation, the extinction coefficients can be converted into standard coefficient multipliers for a 1cm pathlength. Generally, these standard coefficient multipliers are used in place of the extinction coefficient for double-stranded DNA (dsDNA), single-stranded RNA, and single-stranded DNA (Table 1). Using standard coefficient multipliers, the equation for calculating concentration for nucleic acids becomes:

$$\text{Equation 1} \quad \text{Nucleic Acid Concentration} = \frac{\text{OD}_{260}}{\text{Pathlength}} \times \text{Standard Coefficient Multiplier} \times \text{Sample Dilution}$$

Table 1. Standard Coefficients for Nucleic Acids Measured in a 1cm Cuvette.

Molecule	Extinction Coefficient ($\mu\text{g/ml cm}^{-1}$)	1cm pathlength Standard Coefficient ($\mu\text{g/ml}$)
Double-Stranded DNA	0.020	50
Single-Stranded RNA	0.025	40
Single-Stranded DNA	0.027	33

While direct concentration of nucleic acids is fairly accurate, there can be dramatic variation in direct protein concentration results measured at OD₂₈₀. Because only tryptophan, tyrosine and cysteine contribute significantly to protein absorbance at 280nm, the light absorption of protein is dependent

upon the particular amino acid concentration of that protein. However, it should be noted that other aspects of the protein solution may affect the OD₂₈₀ such as protein conformation, protein modifications, buffer types, ionic strength and pH.

In addition, buffer type, ionic strength and pH affect absorptive values and even pure protein solutions may have different conformations. For OD₂₈₀ based protein concentration calculations, there are many methods to empirically derive the extinction coefficient for the protein of interest. However, these methods may not be practical or necessary for most routine lab functions. A very rough protein concentration can be obtained by making the assumption that the protein sample has an extinction coefficient of 1, so 1 OD = 1mg/ml protein (2). For better accuracy, some standard protein extinction coefficients have been published. See Table 2 for a few selected extinction coefficients or the Practical Handbook of Biochemistry and Molecular Biology for a more extensive table [2]. Finally, if the protein sequence of the protein to be measured is known, the theoretical extinction coefficient can be calculated using the equation $\epsilon = 5690(\#Tryptophans) + 1280(\#Tyrosines) + 60(\#Cysteines)$ [3] or online tools such as [ExpASY Protparam](#). Given a known or calculated extinction coefficient, protein concentration can be calculated using the Beer-Lambert equation.

Equation 2 Protein Concentration = $\frac{OD_{280}}{\text{Extinction coefficient} \times \text{Pathlength}} \times \text{Sample Dilution}$

Table 2. Calculated Extinction Coefficients for Proteins Measured in a 1cm cuvette.

Molecule	Calculated Extinction Coefficient (mg/ml) cm ⁻¹ (2)
BSA	0.66
IgG	1.35
IgM	1.45

PATHLENGTH

For single tube instruments, using a standard 10mm x 10mm cuvette, the light pathlength is fixed at 1cm by the distance between the walls of the cuvette (Figure 1A). Absorbance measurements at 1cm pathlength have been correlated with specific nucleic acid concentrations; for example an OD of 1.0 at 260nm correlates to 50µg/ml of dsDNA (Table 1). When using a 1cm cuvette, the pathlength is 1 and Equation 1 can be simplified to OD x Extinction Coefficient x sample dilution. For example, if an undiluted dsDNA sample measured in a 1cm cuvette gives an OD₂₆₀ value of 0.9 OD, the dsDNA concentration would be calculated as: 0.9 OD × 50 = 45µg/ml DNA

However, when using a microplate instrument, measurements are taken vertically so the distance light travels through a sample varies depending on the volume of liquid in the plate (Figure 1B and C). Therefore, to calculate a nucleic acid concentration using Equation 1, a pathlength correction value must be used to account for the different light pathlength corresponding to the sample volume. For example, if the same dsDNA sample was evaluated in a 96-well plate with a 200µl sample volume, the OD value

might be 0.50. Assuming a pathlength of 0.56cm, the dsDNA concentration would be calculated as:
 $((0.50 \text{ OD})/0.56) \times 50 = 45\mu\text{g/ml DNA}$

By including sample pathlength information in the concentration calculation, both single tube measurements and microplate measurements provide comparable results. Pathlength can be calculated two ways: experimentally or mathematically.

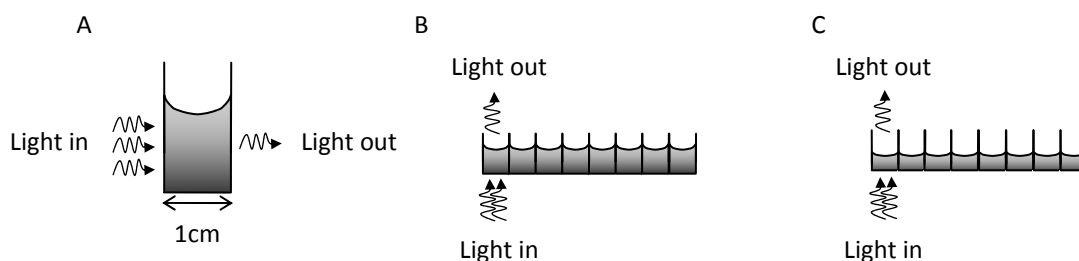


Figure 1. The light pathlength remains constant in the cuvette, assuming the volume is above a minimum threshold, but the pathlength varies in a microplate depending on how much volume is in a well. Light pathlength for: **A.** standard 10mm x 10mm cuvette; **B.** 96-well microplate with 200µl volume; **C.** 96-well microplate with 100µl volume.

Experimentally-Derived Pathlength

Experimentally derived pathlengths are determined by using the absorbance properties of water at 900nm and 977nm wavelengths. While water does not typically absorb light, it does have a small absorbance peak at 977nm. In a 1cm cuvette, the difference in OD at 977nm and 900nm (977nm-900nm) is approximately 0.18 at room temperature. Comparing this standard measurement with the OD values of water at 900nm and 977nm in a microplate allows calculation of the microplate sample pathlength using the following equation.

$$\text{Equation 3} \quad \frac{\text{OD}_{977\text{water}} - \text{OD}_{900\text{water}}}{0.18 \text{ OD}_{1\text{cm cuvette water}}} = \text{Sample Pathlength (cm)}$$

Using filters for 900nm and 980nm and the sample pathlength equation, GloMax®-Multi+ pathlength values for 100 and 200µl microplate volumes in a Corning 96-well UV compatible plate (Corning Cat.# 3635) have been experimentally derived (Table 3).

Table 3. Pathlength Correction Values Calculated using 980nm and 900nm Water Measurements in Corning 96-well UV Compatible Plates (Corning Cat.# 3635).

Sample volume	Pathlength (cm)
100µl	0.29
200µl	0.56

Mathematically-Derived Pathlength

Mathematically, pathlength values can be calculated using the sample volume and the diameter or height and width of the sample plate wells. Microplates have either circular (96-well plates) or square (384-well plates) wells. Using the formulas in Figure 2, the height (pathlength) of the sample volume can

be calculated. It should be noted that pathlengths derived mathematically are approximations, as: 1) the calculation does not take into account the meniscus of the liquid; and 2) the mean diameter of the microplate wells are only estimates as the wells have a slight taper.

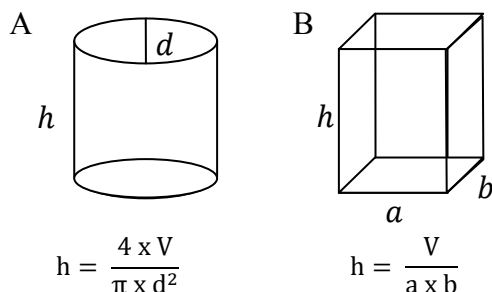


Figure 2. Calculating pathlength using well geometry. A. Calculation of pathlength (h) in plates with cylindrical wells. Where V = sample volume and d = mean diameter of the well. **B.** Calculation of pathlength (h) in plates with square wells. Where V = sample volume, a = mean width of the well and b = mean depth of the well.

Table 4 provides mathematically-derived pathlengths for samples volumes of 25-250 μ l in several common 96- and 384-well microplates. Because pathlength values are proportional to the volume of liquid used, a linear regression has been calculated and can be used to determine the pathlength of any volume between 50 and 250 μ l where x = volume used and y = pathlength. Once the pathlength correction is determined, DNA concentration in a microplate is calculated using Equation 1.

Note: Recommended sample volumes for 96-well plates are 100 μ l-250 μ l. Recommended sample volumes for 384-well plates are 20 μ l and 100 μ l.

Table 4. Pathlength Values and Linear Regression Equation for Various Microplate Well Volumes.

UV compatible plates	Part Number	Pathlength (cm)					Linear Regression
		25 μ l	50 μ l	100 μ l	200 μ l	250 μ l	
BD Falcon 96-well UV plate	353261	n/a	n/a	0.28	0.56	0.70	$y = 0.0028x$
BD Falcon 384-well UV plate	353262	0.19	0.39	0.77	n/a	n/a	$y = 0.0077x$
Corning 96-well UV plate	3635	n/a	n/a	0.29	0.58	0.73	$y = 0.0029x$
Corning 384-well UV plate	3675	0.25	0.50	1.01	n/a	n/a	$y = .0101x$
Corning 96-well half volume UV plate	3679	0.14	0.28	0.56	n/a	n/a	$y = 0.0056x$
Greiner 96-well UV Star (also Thermo Scientific/Nunc)	655801	n/a	n/a	0.28	0.56	0.69	$y = 0.0028x$
Greiner 384-well UV Star (also Thermo Scientific/Nunc)	781801	0.20	0.41	0.82	n/a	n/a	$y = 0.0082x$

Comparing pathlengths for Corning 96-well UV plates (Corning Cat.# 3635) derived from the two methods shows that while the values for 200µl pathlength are slightly different, the overall results are similar regardless of which method is used (Table 5).

Table 5. Experimentally Versus Mathematically -derived Pathlength Values in Corning 96-well UV-Compatible Plates.

Sample volume	Experimentally derived Pathlength (cm)	Concentration of dsDNA with OD of 0.9	Mathematically derived Pathlength (cm)	Concentration of dsDNA with OD of 0.9
100µl	0.29	155µg/ml	0.29	155µg/ml
200µl	0.56	80µg/ml	0.58	78µg/ml

One caveat of using absorbance-based measurements of nucleic acid (RNA and DNA) samples is that proteins and reagents commonly used in the preparation of nucleic acids also absorb light at 260nm and can lead to falsely elevated concentration results. Most reagents that may contaminate a sample also absorb light at 280nm, which provides a method of calculating DNA or RNA purity using the ratio of measurements at OD_{260}/OD_{280} . An OD_{260}/OD_{280} ratio ≥ 1.8 indicates “pure” DNA and an OD ratio of ~ 2.0 indicates “pure” RNA. A ratio below 1.8 indicates DNA or RNA that is contaminated by protein, phenol, or other aromatic compounds. The OD_{260}/OD_{280} ratio does not necessarily indicate the absence of contaminating nucleotides or single-stranded nucleic acids. For protein samples, the converse is true; if the sample is contaminated with nucleic acids, the OD_{260} value will be elevated so that a ratio of OD_{260}/OD_{280} of < 1.0 indicates “pure” protein where as a higher value indicates nucleic acid contamination.

PROTOCOL: QUANTITATING NUCLEIC ACID USING THE GLOMAX®- MULTI+ DETECTION SYSTEM

1. Measure the DNA or RNA sample at both 260nm and 280nm wavelengths using the dual wavelength measurement feature on the GloMax®-Multi+ Detection System.
2. Calculate Nucleic Acid Concentration:
 - a. Subtract the blank value (well containing buffer) from the sample OD_{260} and OD_{280} values.
 - b. Divide the OD_{260} reading by the Table 4 pathlength value corresponding to the volume in the wells.
 - i. If a measurement volume not listed in Table 4 is used, calculate the pathlength correction using the linear regression equation shown in Table 4, if your microplate is listed there, or has similar dimensions to those in Table 4. Otherwise, use Figure 2 to calculate the pathlength.
 - c. Multiply this number by the DNA or RNA constant from Table 1.
 - d. Multiply by the sample dilution factor (if sample was diluted).
3. Estimate nucleic acid purity.
 - a. Subtract the blank values (well containing buffer) from the sample OD_{260} and OD_{280} values.
 - b. Divide the OD_{260}/OD_{280} .

- c. If the ratio of DNA OD_{260}/OD_{280} is between 1.8 and 2.0, it is generally accepted that the DNA purity (free from protein contaminants) is 90% or better. If the ratio of RNA OD_{260}/OD_{280} is ~ 2.0 , it is generally accepted that the RNA purity is 90% or better.

Note: Low OD_{260}/OD_{280} values are often seen when little or no nucleic acid is present.

PROTOCOL: QUANTITATING PROTEIN USING THE GLOMAX®-MULTI+ DETECTION SYSTEM

1. Measure the protein sample at both 280nm and 260nm wavelengths using the dual wavelength measurement feature on the GloMax®-Multi+ Detection System.
2. Calculate Protein Concentration:
 - a. Subtract the blank value (well containing buffer) from the sample OD_{260} and OD_{280} values.
 - b. Divide the OD_{280} reading by the Table 4 pathlength value corresponding to the volume in the wells, and multiply by the extinction coefficient (Table 2, or calculated, or a value of 1 if no value can be determined).
 - i. If a measurement volume not listed in Table 4 is used, calculate the pathlength correction using the linear regression equation shown in Table 4, if your microplate is listed or your plate has similar well dimensions as those listed in the table. Otherwise, calculate the pathlength using the equations in Figure 2.
 - c. Multiply by the sample dilution factor (if sample was diluted).
3. Estimate nucleic acid contamination:
 - a. Subtract the blank values (well containing buffer) from the sample OD_{260} and OD_{280} values.
 - b. Divide the OD_{260}/OD_{280} .
 - c. It is generally accepted that if the ratio of DNA OD_{260}/OD_{280} is < 1.0 , the protein purity (free from nucleic acid contaminants) is $\sim 95\%$ or better.

REFERENCES

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3. Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**,319-26.

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