Tech Note

DNA Quantification using Gen5™

Peter J. Brescia and Peter Banks, Applications Department, BioTek Instruments, Inc., Winooski, VT

This technical note describes methods for performing dsDNA quantification with BioTek microplate readers and Gen5™ software. The methods are useful with BioCell™, microplates or Take3™ Micro-Volume Plate. An overview of the calculations made, the details of the calculations, and a discussion of Gen5 actions are presented.

Introduction

The quantification of nucleic acids from a variety of sources, in particular dsDNA, is commonly accomplished by using ultra-violet (UV) spectrophotometry. Traditional measurements have been done in a cuvette-based spectrophotometer with a 1 cm path length vessel in accordance with the Beer-Lambert Law:

A = εlc, where A is the measured absorbance, ε is the extinction coefficient of the analyte, l is the path length of the measurement and c is the concentration of absorbing species.

For the quantification of dsDNA an average mass extinction coefficient, ε_{dsDNA} is typically used. ε_{dsDNA} is equal to 0.020 $(\mu g/mL)^{-1}cm^{-1}$, where an optical density (OD) measurement equal to 1 absorbance unit (A_{260} or OD = 1) in a 1 cm path length vessel results in the following:

$$c = \frac{A}{\varepsilon \cdot l} = \frac{1\ OD}{0.020\ (\mu g/mL)^{-1} cm^{-1} \cdot 1\ cm} = 50\ \mu g/mL$$

For simplicity, 50 µg /mL/OD is commonly used with 1 cm path length vessels to compute DNA concentrations by multiplying the measured absorbance by 50:

$$c = 50 \cdot A$$

DNA quantification can also be performed in a microplate reader to process many more samples than a cuvette spectrophotometer. The principal challenge with microplate-based analysis is that the path length of measurement in a microplate is not fixed by the vessel, but by the volume of solution in the well, which can vary. There are methods available to normalize measurements to a 1 cm path length that will be discussed later. Various accessories have been developed to provide fixed path lengths for use in microplate readers, such as those that provide 1 cm path lengths (BioCell or stoppered cuvettes) and also those that allow for micro-volume analysis (Take3 Plate) – see Figure 1. Micro-volume analysis in particular is a very useful procedure for DNA quantification because the short path length allows for analysis without the need for any dilution of samples.

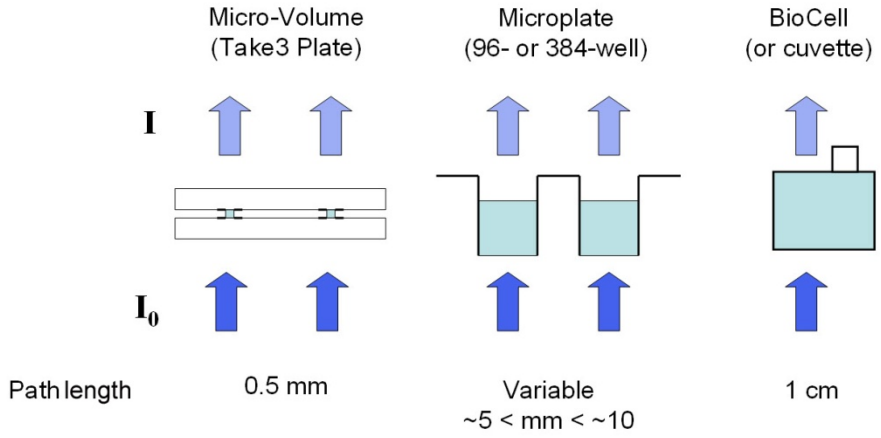

Figure 1. Multi-Volume analysis capabilities in a microplate reader.

In this technical note, we will describe the calculations and corrections typically used for the accurate quantification of dsDNA. We will demonstrate those appropriate for 1 cm path length measurements in a BioCell (cuvette), variable path length measurements in a microplate and micro-volume measurements using the Take3 or Take3 Trio plate. For clarity purposes, we will provide a brief overview of the calculations made first, followed by detailed calculations in a separate section.

Overview

BioCell (or cuvette)

Measurements are commonly performed at wavelengths of 260, 280 and 320 nm. A_{260} is the preferred wavelength for nucleic acid quantification. A_{280} measurements provide a means for the assessment of nucleic acid purity, which is commonly done on freshly isolated samples as a quality control measure. Finally, A_{320} measurements are made to correct for light scatter associated with insoluble particulates in suspension which may originate from the isolation process. In addition to these measurements made on the sample, the same three measurements are made on a blank, which contains the same volume and solvent as the sample.

Thus, the steps for accurate quantification using a BioCell are:

1. Obtain raw optical density measurements at all three wavelengths on Blank
2. Obtain raw optical density measurements at all three wavelengths on Sample
3. Subtract the A_{320} measurements from Sample and Blank
4. Perform Blank subtraction
5. Assess nucleic acid purity by taking the ratio of corrected A_{260}/A_{280}: ratios of 1.8 – 2.0 are indicative of high purity nucleic acid
6. Compute concentration of the sample by multiplying the corrected A_{260} measurement by 50

Microplate

Microplates are typically used to process multiple samples. Many microplate manufacturers offer UV-transparent plates, ideal for nucleic acid quantification. Blank subtraction should be performed for each well of the microplate as imperfections caused in plate manufacturing can interfere with results. For simplicity, these blank measurements are made with an empty plate before a dding samples. Recall that the path length in microplate measurements is determined by the volume of solution contained in the well. Microplate analysis using Gen5 software takes advantage of a small but measurable peak absorbance of water at 977 nm. Measurements at 900 nm are used for background correction. The ratio of the sample measurement to standard absorbance of water at 1 cm is used to determine each well's path length.

This is considered a constant and Gen5 uses the value 0.18 to represent the 1 cm path length case. Thus, path length can be computed according to the following equation:

$$\text{Path length} = \frac{A_{977} - A_{900}}{0.18}$$

Sample measurements can then be path length corrected by dividing them by the computed path length. This essentially normalizes the data to a 1 cm path length.

Thus the steps for accurate quantification using a microplate and Gen5 software are:

1. Obtain Blank measurements at A_{260} and A_{280} using an empty microplate
2. Obtain raw optical density measurements on samples at A_{260}, A_{280}, A_{900} and A_{977}
3. Compute path length for each sample well (done automatically by Gen5)
4. Perform Blank subtraction
5. Assess nucleic acid purity by taking the ratio of corrected A_{260}/A_{280}: ratios of 1.8 – 2.0 are indicative of high purity nucleic acid
6. Compute path length corrected results (done automatically by Gen5)
7. Compute concentration of the sample by multiplying the corrected A_{260} measurement by 50

Take3 Micro-Volume Plate

The Take3 accessory has a nominal path length of 0.5 mm as defined by the two quartz slides (see Figure 1). Calibration of exact path lengths is performed during manufacture and imported into the Gen5 software. Recalibration can be performed by Gen5 software if needed using the procedure described in the user's manual. There are two options for Blanking. The recommended method is to use each microwell for Blanking by first measuring the Blank, cleaning the microwell, then adding Sample and remeasuring. This is termed *Well-to-Well Blanking*. A second method is to dedicate a number of microwells for Blanking and the rest for Samples. This method is termed *Blank Average*. This method has the advantage of rapid analysis (no need to have separate Blank and Sample loads), but can result in slightly less accurate readings than Well-to-Well Blanking. There are also two options for reporting optical density readings: either normalized to 0.5 mm path length; or to 1 cm path length.

Gen5 automates the read steps and calculations required for accurate quantification using the Take3 plate.

With *Well-to-Well Blanking*:

1. Obtain raw optical density measurements at A_{260}, A_{280} and A_{320} on Blank
2. Obtain raw optical density measurements at A_{260}, A_{280} and A_{320} on Sample
3. Subtract the A_{320} measurements from Sample and Blank
4. Perform Blank subtraction
5. Assess nucleic acid purity by taking the ratio of corrected A_{260}/A_{280}: ratios of 1.8 – 2.0 indicate high purity nucleic acid
6. Normalize corrected A_{260} to 0.05 or 1 cm path length (optional)
7. Compute concentration of the sample by multiplying the corrected A_{260} measurement by 20 * 50 or 50 for 0.05 or 1 cm path length normalized measurements, respectively.

With *Blank Average*:

1. Obtain raw optical density measurements at A_{260}, A_{280} and A_{320} on both Sample and Blank microwells
2. Subtract the A_{320} measurements from Sample and Blank
3. Perform Blank subtraction
4. Assess nucleic acid purity by taking the ratio of corrected A_{260}/A_{280}: ratios of 1.8 – 2.0 indicate high purity nucleic acid
5. Normalize corrected A_{260} to 0.05 or 1 cm path length (optional)
6. Compute concentration of the sample by multiplying the corrected A_{260} measurement by 20 * 50 or 50 for 0.05 or 1 cm path length normalized measurements, respectively.

Calculations

Note:

Many calculations are automatically performed by Gen5, including blank subtraction and path length correction (for microplate measurements).

BioCell: Standard 1 cm path length

Collect Raw Data for each sample well

(Steps 1 and 2):

$A_{Blank:260}$, $A_{Blank:280}$, $A_{Blank:320}$
$A_{Samp:260}$, $A_{Samp:280}$, $A_{Samp:320}$

Compute Bichromatic Results for each sample well (Step 3)

$a_{Blank:260} = A_{Blank:260} - A_{Blank:320}$
$a_{Blank:280} = A_{Blank:280} - A_{Blank:320}$
$a_{Samp:260} = A_{Samp:260} - A_{Samp:320}$
$a_{Samp:280} = A_{Samp:280} - A_{Samp:320}$

Compute Blank Corrected Results for each sample well (Step 4)

$\alpha_{Samp:260} = a_{Samp:260} - a_{Blank:260}$
$\alpha_{Samp:280} = a_{Samp:280} - a_{Blank:280}$

Compute Ratio for each sample well (Step 5)

The ratio is based on buffer corrected results.

$$Ratio_{260/280} = \alpha_{Samp:260} / \alpha_{Samp:280}$$

Compute Concentration for each sample well (Step 6)

$$Conc_{Samp} = \alpha_{Samp:260} * 50$$

Microplate: Variable path length

The following calculations are required when making determinations using a microplate with path length correction.

Collect Raw Data for each sample well (Steps 1 and 2)

$A_{Blank:260}$, $A_{Blank:280\ (empty\ plate)}$
$A_{Samp:260}$, $A_{Samp:280}$, $A_{Samp:900}$, $A_{Samp:977}$

Compute Path length for each sample well (Step 3)

$$Path\ length_{Sample}\ well = (A_{900} - A_{977})well/(A_{900} - A_{977})1.0\ cm\ water$$

Gen5 uses the default value of 0.18 for the absorbance of water at 1 cm.

Compute Blank Corrected Results for each sample well (Step 4)

$a_{Samp:260} = A_{Samp:260} - A_{Blank:260}$
$a_{Samp:280} = A_{Samp:280} - A_{Blank:280}$

Compute Ratio (Step 5)

$Ratio_{260/280} = a_{Samp:260} / a_{Samp:280}$

Compute Path length Corrected Results (1 cm) (Step 6)

$\alpha'_{Samp:260} = a_{Samp:260} / \text{Path length}_{Sample\ well}$

Concentrations

$Conc_{Samp:260} = \alpha'_{Samp:260} * 50$

Take3: Nominal 0.5 mm Path Length with Well-to-Well Blanking

The following calculations are performed by Gen5 when well-to-well blanking is used for determinations using Take3 micro-volume measurements.

Collect Raw Data for each sample well (Steps 1 and 2)

$A_{Blank:260}$, $A_{Blank:280}$, $A_{Blank:320}$
$A_{Samp:260}$, $A_{Samp:280}$, $A_{Samp:320}$

Compute Bichromatic Results for each sample well (Step 3)

$a_{Blank:260} = A_{Blank:260} - A_{Blank:320}$
$a_{Blank:280} = A_{Blank:280} - A_{Blank:320}$
$a_{Samp:260} = A_{Samp:260} - A_{Samp:320}$
$a_{Samp:280} = A_{Samp:280} - A_{Samp:320}$

Compute Blank Corrected Results for each sample well (Step 4)

$\alpha_{Samp:260} = a_{Samp:260} - a_{Blank:260}$
$\alpha_{Samp:280} = a_{Samp:280} - a_{Blank:280}$

Compute Ratio for each sample well (Step 5)

$Ratio_{260/280} = \alpha_{Samp:260} / \alpha_{Samp:280}$

(Optional) Compute Path length Corrected Results (0.5 mm) for each sample well (Step 6 option)
Perform this step if the preference is to normalize results to 0.5 mm (see preferences below).

$\alpha'_{Samp:260} = \alpha_{Samp:260} * 0.5 / \text{Path length}_{Sample\ well}$
$\alpha'_{Samp:280} = \alpha_{Samp:280} * 0.5 / \text{Path length}_{Sample\ well}$

(Optional) Compute Path length Corrected Results (1 cm) for each sample well (Step 6 option)
Perform this step if the preference is to normalize results to 1 cm (see preferences below).

$\alpha'_{Samp:260} = \alpha_{Samp:260} * 0.5 / \text{Path length}_{Sample\ well} * 20$
$\alpha'_{Samp:280} = \alpha_{Samp:280} * 0.5 / \text{Path length}_{Sample\ well} * 20$

Compute Concentration for each sample well (Step 7)

$Conc_{Samp} = \alpha'_{Samp:260} * 50 * 20$ (if 0.5mm corrected data)
$Conc_{Samp} = \alpha'_{Samp:260} * 50$ (if 1cm corrected data)

Take3: Nominal 0.5 mm path length with Blank Average

The following calculations are performed by Gen5 when a blank average is used for determinations using Take3 micro-volume measurements.

Collect Raw Data for all blanks (Step 1 for Blanks)

$A_{Blank\ 1:260}, \ldots A_{Blank\ n:260}$
$A_{Blank\ 1:280}, \ldots A_{Blank\ n:280}$
$A_{Blank\ 1:320}, \ldots A_{Blank\ n:320}$

Collect Raw Sample Data for each sample well (Step 1 for Samples)

$A_{Samp:260}, A_{Samp:280}, A_{Samp:320}$

Compute Bichromatic Results for all blank wells (Step 2 for Blanks)

$a_{Blank\ n:260n} = A_{Blank\ n:260} - A_{Blank\ n:320}$
$a_{Blank\ n:280n} = A_{Blank\ n:280} - A_{Blank\ n:320}$

Compute Bichromatic Results for each sample well (Step 2 for Samples)

$a_{Samp:260} = A_{Samp:260} - A_{Samp:320}$
$a_{Samp:280} = A_{Samp:280} - A_{Samp:320}$

Compute Average Bichromatic Results for blank wells (Step 3)

$a_{Blank\ Ave:260} = AVG\ (a_{Blank\ 1:260 \ldots Blank\ n:260})$
$a_{Blank\ Ave:280} = AVG\ (a_{Blank\ 1:280 \ldots Blank\ n:280})$

Compute Blank Corrected Results (Step 3)

$\alpha_{Samp:260} = a_{Samp:260} - a_{Blank\ Ave:260}$
$\alpha_{Samp:280} = a_{Samp:280} - a_{Blank\ Ave:280}$

Compute Ratio (Step 4)

$Ratio_{260/280} = \alpha_{Samp:260} / \alpha_{Samp:280}$

(Optional) Path length Corrected Results (0.5 mm) (Step 5 option)
Perform this step if the preference is to normalize results to 0.5 mm (see preferences below).

$\alpha'_{Samp:260} = \alpha_{Samp:260} * 0.5 / Path\ length_{Sample\ well}$
$\alpha'_{Samp:280} = \alpha_{Samp:280} * 0.5 / Path\ length_{Sample\ well}$

(Optional) Path length Corrected Results (1 cm) (Step 5 option)
Perform this step if the preference is to normalize results to 1 cm (see preferences below).

$\alpha'_{Samp:260} = \alpha_{Samp:260} * 0.5 / Path\ length_{Sample\ well} * 20$
$\alpha'_{Samp:280} = \alpha_{Samp:280} * 0.5 / Path\ length_{Sample\ well} * 20$

Concentrations

$Conc_{Samp:260} = \alpha'_{Samp:260} * 50 * 20$ (if 0.5mm corrected data)
$Conc_{Samp:260} = \alpha'_{Samp:260} * 50$ (if 1cm corrected data)

Gen5 Steps for measurements using Take3 Plate

Measurement of nucleic acids samples using BioCell, cuvette or microvolume analysis with Take3 plate and Gen5 software are accomplished via the Gen5 interface by selecting Nucleic Acid Quantification under the Take3 Applications in the Task Manager window (Figure 2).

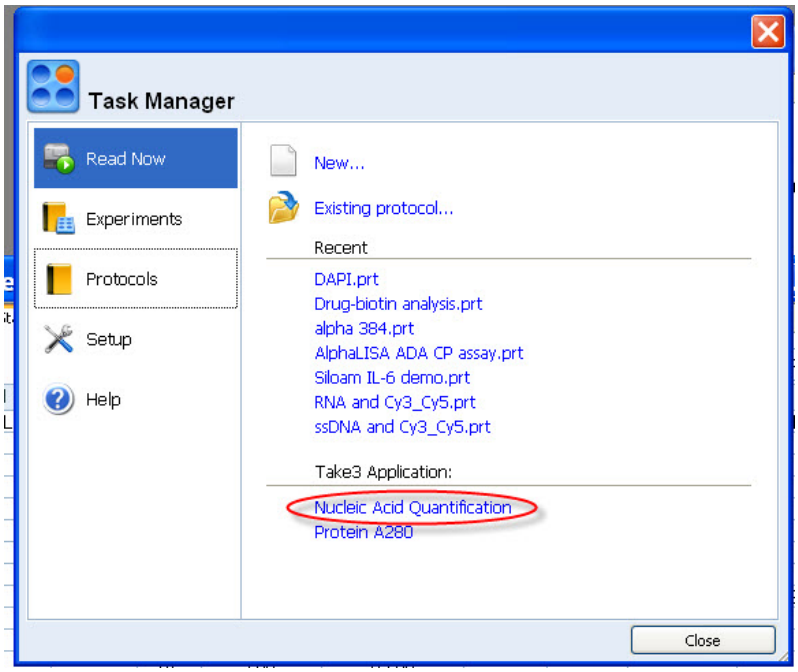

Figure 2: Quantification of dsDNA in cuvette, BioCell or micro-volume in Gen5 begins by selecting the **Take3 Application:** ***Nucleic Acid Quantification*** from the **Read Now** menu.

The Take3 user interface window allows selection of the **Sample Type** (Step 1) and **Well Type** (Step 2) from the drop-down menus (Figure 3). Once selections are made for the **Well Type** the appropriate read location will be highlighted in the plate well location map (cuvette read locations for the Take3 plate are highlighted light blue)(Figure 3). The default **Sample Type** settings can be viewed and any changes to the sample type can be made by selecting **Nucleic Acid Sample Type** from the **Take3** menu (Step 3), selecting dsDNA and clicking on the View/Modify button (Step 4)(Figure 3). Default read parameters for dsDNA analysis include bichromatic measurements as described above as well as measurement at a reference wavelength of 320 nm. A secondary ratio wavelength can also be selected to gain additional information regarding sample purity, typically 230 nm.

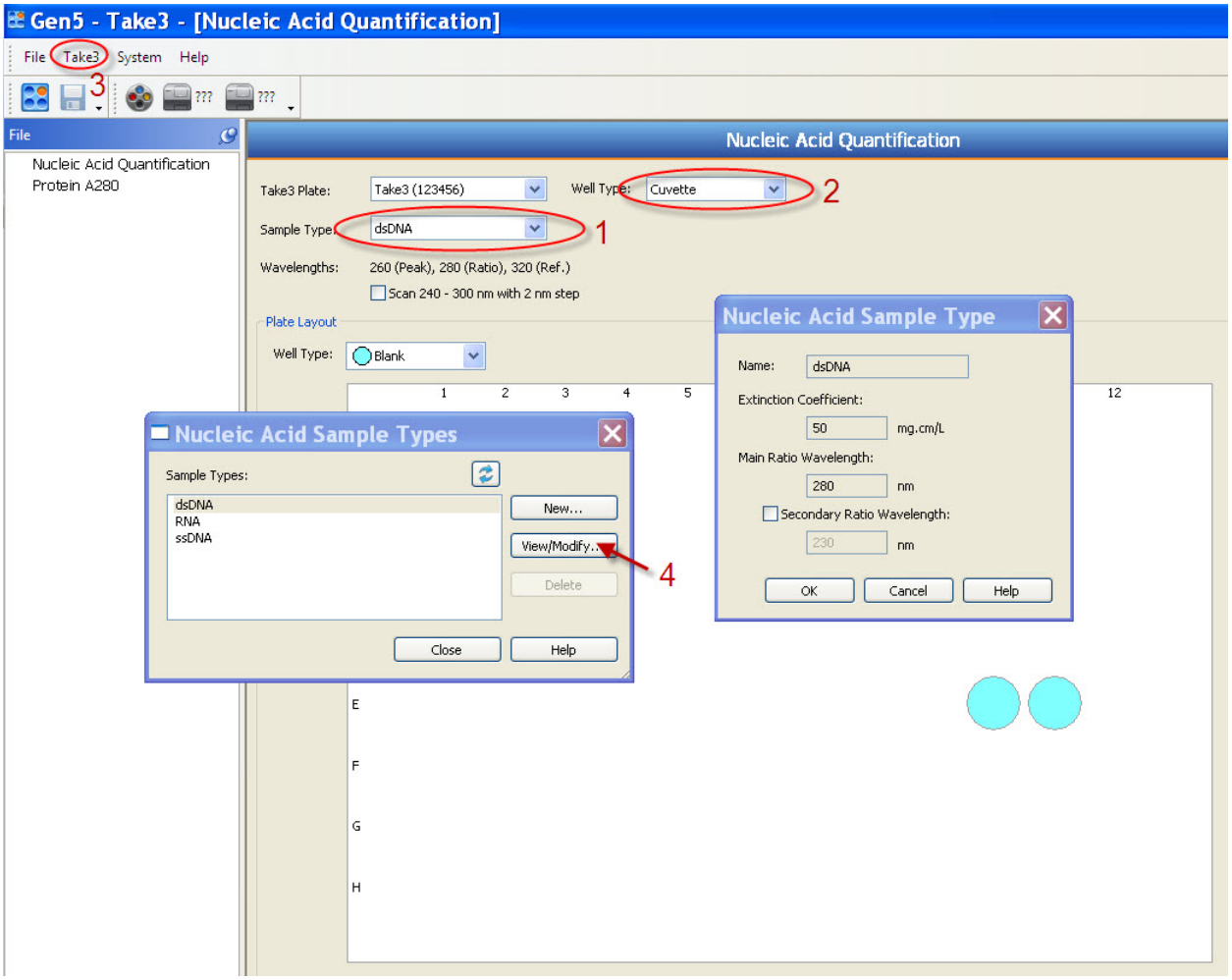

Figure 3: Selection of sample type: dsDNA (1) and Well Type: Cuvette or BioCell™ (2) for 1 cm path length measurements. Selection of Nucleic Acid Sample Type from the Take3 menu (3), selecting dsDNA and clicking the View/Modify button (4) displays the sample type parameters.

Measurement data is exported in Excel format and will vary slightly depending on what vessel type is selected. The measurement is read in two steps with the first of the two read steps providing blanking data of the appropriate solvent prior to reading of the sample (Figure 4). Blanking data is exported to Excel and sample measurements can be taken once a satisfactory value for blanking has been approved (Figure 5).

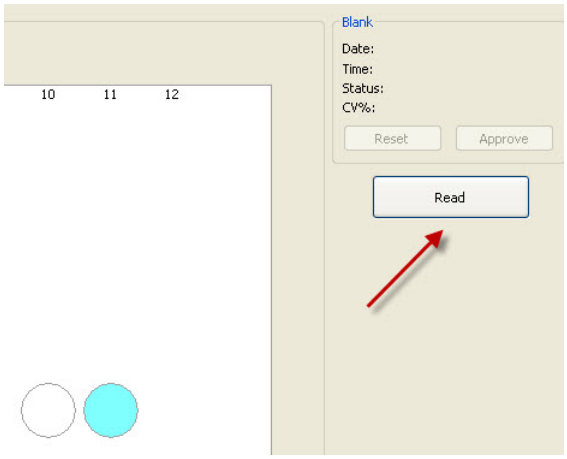

Figure 4: Prior to making sample measurements a blanking step is required.

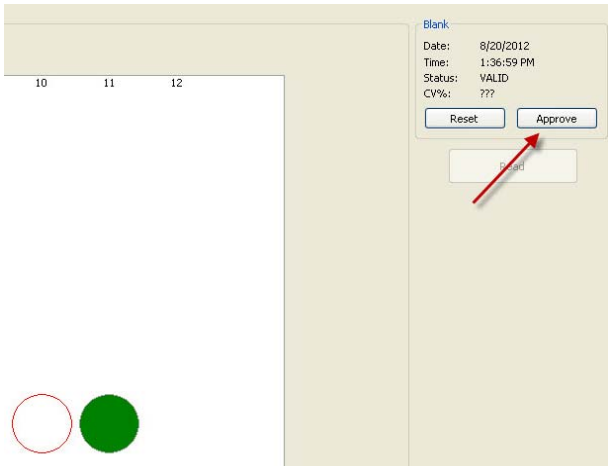

Figure 5: Once the appropriate blanking solution has been read approval of blanking data must be verified by clicking the Approve button prior to reading sample measurements.

Figure 6 is representative of data exported when measuring sample in a cuvette. Data in rows 9-12 represents data that has been blank subtracted using data in row 25 and exported to Excel after sample measurements are taken. The A_{260}/A_{280} ratio is calculated by dividing the blank subtracted values:

$$\frac{A260}{A280} = \frac{0.095}{0.051} = 1.866$$

while the concentration represents the blanked A_{260} value multiplied by the correction factor for dsDNA:

$$c = A260 \times 50\,\frac{ng}{\mu L} = 0.095OD \times 50\,ng/\mu L/OD = 4.74\,ng/\mu L.$$

	A	B	C	D	E	F
1	**Sample Results:**					
2						
3	Sample Type:	dsDNA				
4	Sample Read:	1				
5	Date:	7/30/2009				
6	Time:	2:09:20 PM				
7						
8				9		
9			A	0.095	260	
10				0.051	280	
11				1.866	260/280	
12				4.74	ng/µL	
13						
14	Gen5 version:	1.09.5				
15	Take3 Plate:	116440				
16	Reader Type:	Epoch				
17	Reader Serial Number:	P235026				
18						
19	**Blank Results:**					
20						
21	Date:	7/30/2009				
22	Time:	2:07:58 PM				
23						
24			Location	260 Raw	280 Raw	320 Raw
25			A9	0.062	0.05	0.042
26						

Figure 6: After sample reads data is export to Excel as a new worksheet.

Several consecutive sample reads may be done using the approved blank data. Once all reads have been completed a summary is generated by clicking on the End of Batch button in the Gen5™ software interface (Figure 7).

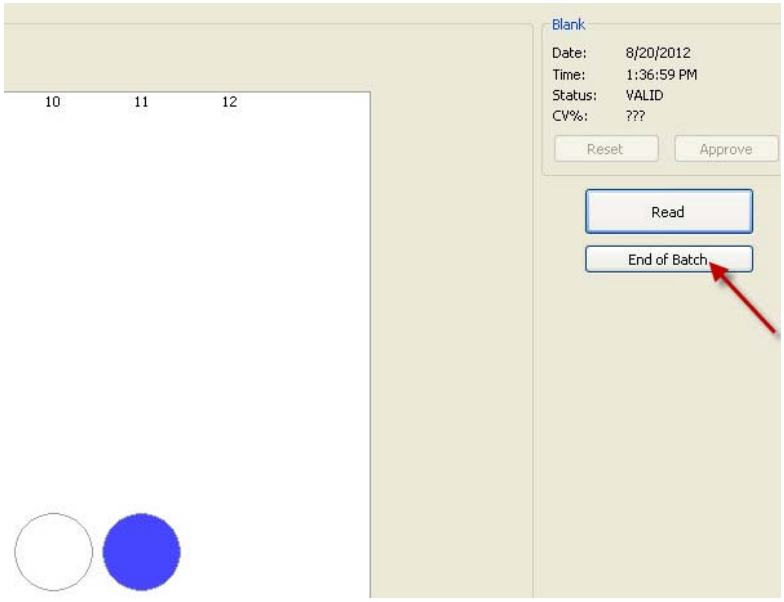

Figure 7: Selecting End of Batch ends the read session and exports a summary of all blank and sample reads occurring during the session.

Batch results contain both raw measurement data as well as blanked, path length corrected data normalized to the user selected preference (0.5 mm or 1 cm), as well as concentration determinants (Figure 8).

Sample Results:

Sample Read#	Location	260 Raw	280 Raw	320 Raw	260	280	260/280	ng/µL
1	A9	0.157	0.101	0.042	0.095	0.051	1.866	4.74
2	A9	0.25	0.151	0.043	0.186	0.099	1.873	9.3
3	A9	0.424	0.244	0.042	0.362	0.194	1.867	18.12
4	A9	0.776	0.432	0.042	0.714	0.381	1.871	35.695
5	A9	1.465	0.799	0.043	1.402	0.748	1.873	70.075
6	A9	2.778	1.515	0.045	2.712	1.462	1.855	135.62
7	A9	0.328	0.192	0.041	0.267	0.143	1.869	13.335
8	A9	0.591	0.333	0.041	0.53	0.284	1.867	26.5
9	A9	1.115	0.612	0.043	1.052	0.562	1.872	52.585
10	A9	2.113	1.145	0.042	2.05	1.095	1.872	102.525
11	A9	3.12	1.714	0.043	3.057	1.663	1.838	152.84
12	A9	1.299	0.711	0.041	1.237	0.662	1.87	61.85
13	A9	1.736	0.943	0.042	1.674	0.893	1.874	83.72

Figure 8: Sample data from dsDNA measurements made using a cuvette.

Gen5™ Steps for Microplate Measurements

Microplate measurements are taken using a standard Gen5 protocol setup with read steps as shown in figure 9. The first read step is a pre-read to collect data for blank subtraction as discussed above. The plate is then ejected for plate loading of sample followed by absorbance measurements at 260 and 280 nm.

Note: Path length correction is selected in the second read step for calculation of the variable path length as described above (Figure 10).

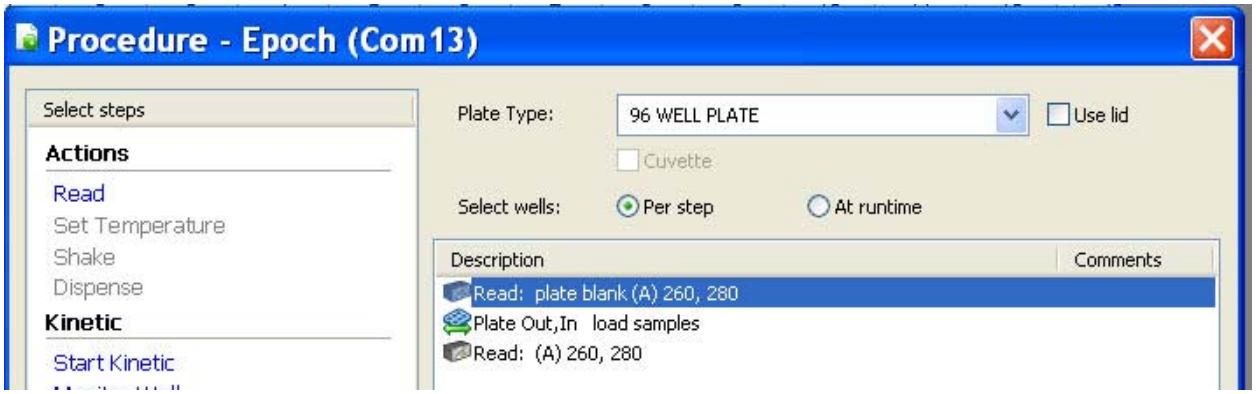

Figure 9: During microplate analysis of dsDNA samples the plate is first pre-read for blanking followed by sample loading and measurements at 260 and 280 nm using a standards Gen5 protocol.

Figure 10: During sample measurements path length correction is selected for determination of the variable path lengths inherent with microplate measurements.

Data reduction steps include blank subtraction of pre-read data for both the 260 and 280 nm measurements as shown in detail in figure 11 for the 260 nm measurement.

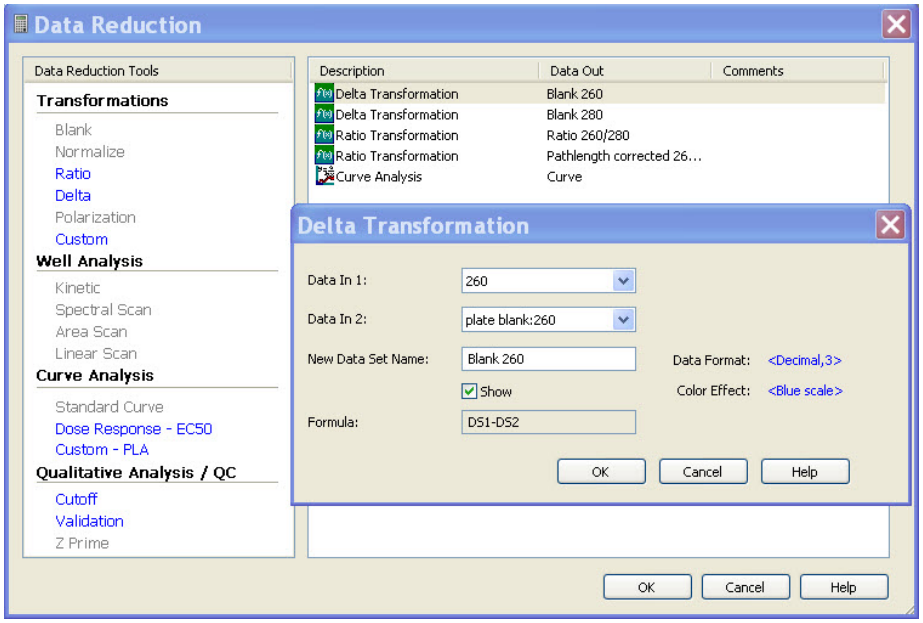

Figure 11: Representative dialogue box in Gen5 software data reduction for the delta transformation for blank subtraction of the A_{260} measurement.

The A_{260}/A_{280} ratio can then be determined for each sample using the transformation depicted in Figure 12 as it is independent of path length.

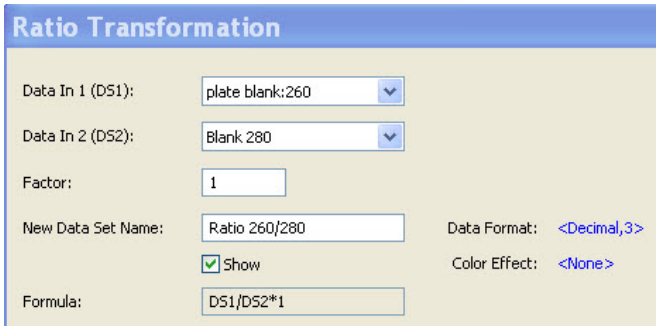

Figure 12: Representative dialogue box in Gen5 software data reduction for the ratio transformation for determination of the A_{260}/A_{280} ratio for assessment of sample purity.

Path length corrected A_{260} is then calculated by determining the ratio of the Blank A_{260} data to the path length determined by the Gen5™ software (Figure 13).

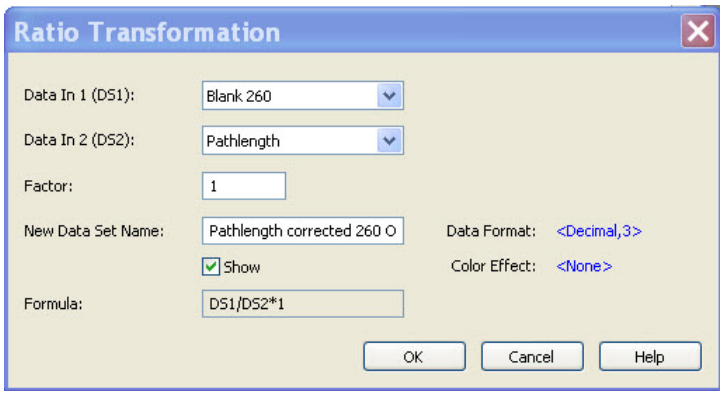

Figure 13: The ratio of Blank 260 data divided by the path length determined by the Gen5 software path length correction function is used to normalize data to a 1 cm path length measurement.

The path length corrected A_{260} values are then used in a final data reduction transformation step in conjunction with the correction factor for dsDNA to determine the sample concentration (Figure 14).

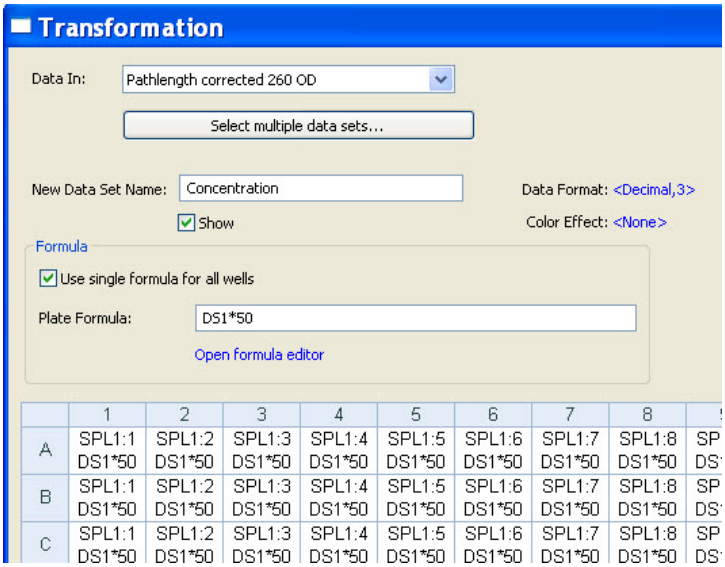

Figure 14: Path length corrected A_{260} measurements are multiplied by the correction factor 50 µg/mL/OD for derivation of the sample concentrations.