

# Microplate Based Pathlength Correction Method for Photometric DNA Quantification Assay

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## Abstract

This technical note describes how to use a simple photometric pathlength correction method to calculate nucleic acid concentrations from sample absorbance measured on a microplate with the Thermo Scientific Multiskan GO spectrophotometer.

Pathlength correction normalizes absorbance values measured on a microplate to correspond to absorbance values measured in a standard cuvette. The liquid pathlength in a standard cuvette is 1 cm, whereas the liquid pathlength in a microplate is not fixed. On microplates the absorbance is measured vertically through the well, so several factors affect the liquid pathlength and thus the absorbance. Therefore in photometric microplate measurements pathlength correction is required for calculating the DNA/RNA concentrations directly from the absorbance values.

For example,  $A_{260\text{nm}}$  1.0 of dsDNA measured at a 1 cm pathlength corresponds to a concentration of 50 µg/ml. This known multiplication factor together with the automatic pathlength correction in SkanIt Software for Multiskan GO spectrophotometer make the DNA analysis with microplates very simple.

## Theory

According to Lambert-Beer's Law the concentration of a compound has a linear correlation to its absorbance at a defined wavelength at a constant pathlength:

$$A = \epsilon * C * d \quad (\text{Function 1. Lambert-Beer's Law})$$

Where:

A = Absorbance

$\epsilon$  = extinction coefficient (molar absorption coefficient)

C = concentration

d = pathlength (in cm)

When A,  $\epsilon$  and d (normally 1.0 cm) are known, the concentration can be calculated:

$$C = \frac{A}{\epsilon * d} \quad (\text{Function 2.})$$

When measuring, for example, nucleic acid (DNA, RNA) absorbance on a microplate, the liquid pathlength is not fixed to 1 cm, so the absorbance values cannot be used as such for concentration calculation. Therefore the true liquid pathlength of each well must be known before Lambert-Beer's Law can be applied to microplate measurements. If the microplate assay includes standards with known concentrations, pathlength correction is not needed.

## Factors affecting the liquid pathlength on a microplate

The liquid pathlength in microplate wells depends mainly on the liquid volume, microplate well dimensions and the meniscus effect of the liquid surface.

The liquid pathlength cannot be calculated directly from the volume intended to pipette into the well. The microplate wells have a conical shape, which makes pathlength calculation complicated. It would also be practically impossible to take the meniscus shape of the liquid surface correctly into account. Therefore the well geometry and meniscus shape cause the measured absorbance to have a very complicated dependence on the liquid volume. The dependence is neither linear nor does it follow any simple mathematical formula. It strongly depends on the microplate type and liquid composition. Figure 1 shows how the liquid pathlength can differ on microplates from different manufacturers even when pipetting the same volume of the same liquid.

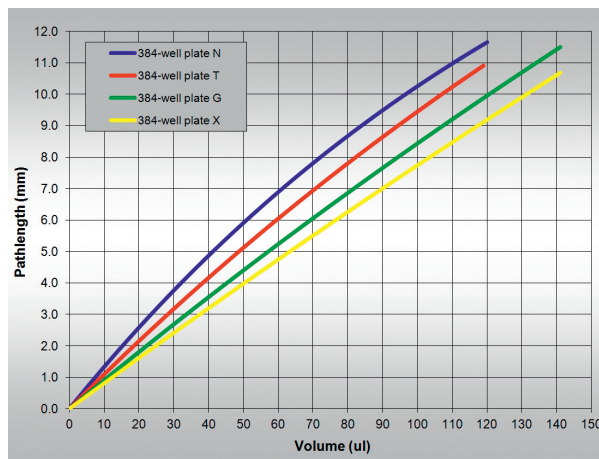


Figure 1. Theoretical dependence of the liquid volume on the liquid pathlength with 384-well microplates of four different manufacturers.

The meniscus effect can also affect the pathlength. The measured analyte itself can affect the strength of the meniscus. For example, in nucleotide solutions (RNA and DNA) the concentration affects the meniscus formation. Figure 2 shows an example of the pathlength changes by RNA concentration even when the liquid volume per well stays the same. With low concentrations the liquid pathlength stays the same as with water. But with concentrations above  $\sim 20 \mu\text{g/ml}$  the meniscus effect starts to shorten the pathlength, and with very high RNA concentrations the pathlength is almost 15% shorter.

Different buffer compositions also change the liquid pathlengths, because the physical characteristics (surface tension, polarity, etc.) of the solution affect the meniscus formation. Examples of the meniscus changes caused by either buffer composition or nucleotide concentration are shown in Figure 3. As a result, there is no reliable way to calculate the liquid pathlength solely by the added volume. The pathlength has to be measured separately from each well and this value used to normalize the measured absorbance to 10 mm pathlength.

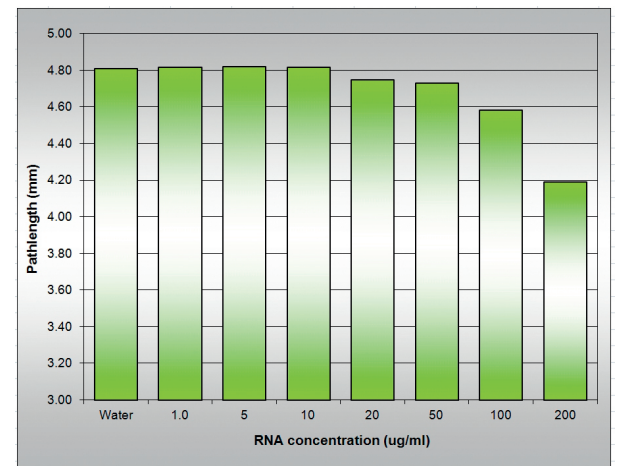


Figure 2. The effect of RNA concentration on the liquid pathlength with a 96-well half area plate ( $75 \mu\text{l}$  RNA solution/well).

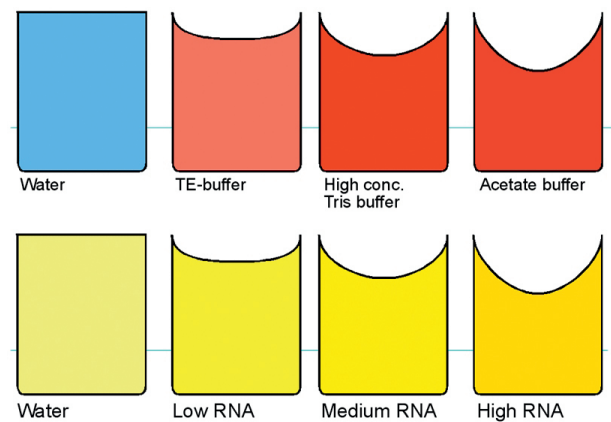


Figure 3. Different solutions can have different meniscus shapes and therefore different pathlengths with exactly the same volume.

## Determining the liquid pathlength

The liquid pathlength on a microplate well can be measured from water absorption in the near IR area. Figure 4 shows the absorption spectrum of water measured with a 10 mm polyacrylic disposable cuvette. The absorption maximum is at 975 nm which is therefore the most optimal wavelength to measure the liquid pathlength. A reference measurement at 900 nm is used to eliminate the background absorbance of the plastic, etc.

When you measure the absorbance of the assay buffer at 975 nm and 900 nm with a 10 mm cuvette, and perform a subtraction  $A_{975} - A_{900}$ , a reference value called the “K-factor” is obtained. The liquid pathlength of each well on a microplate can then be calculated with Function 3. When the actual liquid pathlength has been determined, the corrected absorbance value corresponding to the 10 mm pathlength can be calculated with Function 4.

$$\text{Pathlength} = \frac{A_{975}(\text{well}) - A_{900}(\text{well})}{A_{975}(\text{cuvette}) - A_{900}(\text{cuvette})} * 10\text{mm} = \frac{A_{975}(\text{well}) - A_{900}(\text{well})}{K - \text{factor}} * 10\text{mm}$$

(Function 3)

$$A_{\text{corrected}} = A_{\text{raw}} * \frac{10\text{mm}}{\text{Pathlength}(\text{well})} = A_{\text{raw}} * \frac{K - \text{factor}}{A_{975}(\text{well}) - A_{900}(\text{well})}$$

(Function 4)

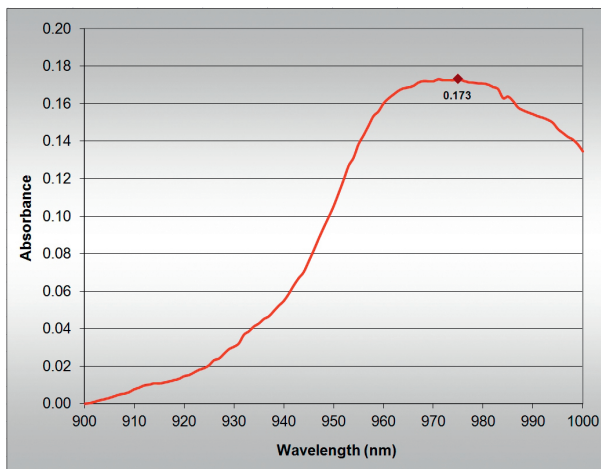


Figure 4. Absorption spectrum of water in the IR area. The raw spectra was subtracted with the reference background value measured at 900 nm. The absorption maximum is at 975 nm. The absorption maximum value 0.173 at 975 nm is the pathlength correction K-factor for pure water.

## Effect of the buffer composition

The K-factor depends on the composition of the assay buffer. The K-factor should therefore be measured with a buffer composition as close as possible to the solution that is going to be used in the actual microplate assays. Examples of K-factors determined with different buffer and solvent compositions are shown in Figure 5. The K-factor is close to 0.17 with water, low concentration buffers and solvents, but when the buffer strength or solvent concentration increases, the K-factor can decrease remarkably.

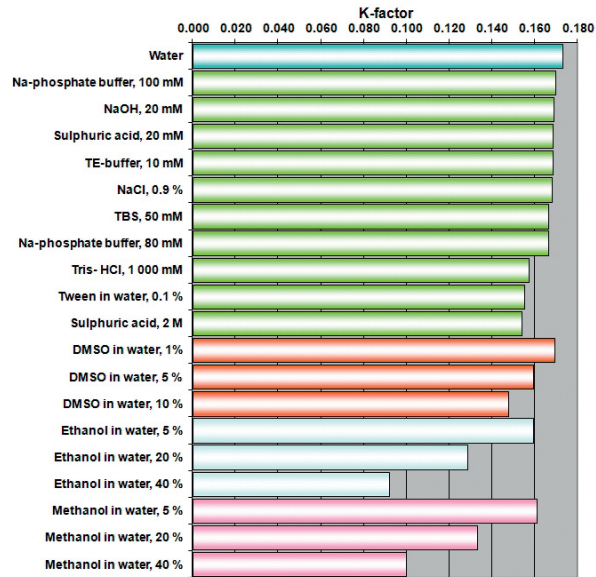


Figure 5. Pathlength correction K-factors of different buffer compositions measured at 25 °C.

## Effect of the temperature

Water absorbance in the near IR area depends on the liquid temperature. The K-factor should be determined with a 10 mm cuvette at the same temperature as the actual microplate assays are going to be performed. The temperature dependence of a water K-factor is shown in Figure 6.

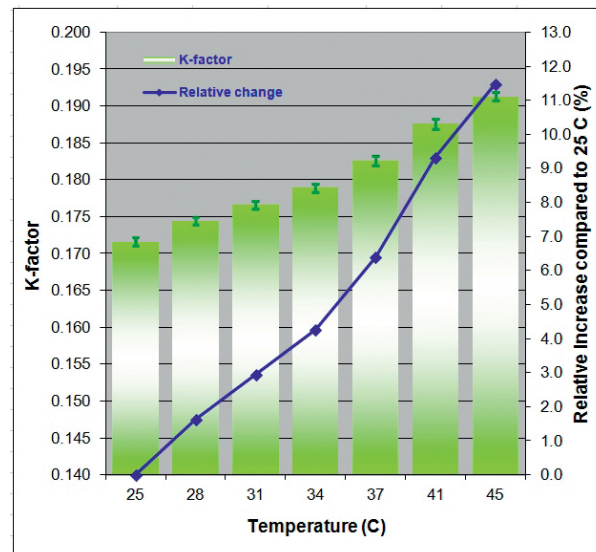


Figure 6. Pathlength correction K-factors of water at different temperatures.

As seen in Figure 6, the water K-factor (absorption of water at 975 nm) increases when the temperature increases. About a ten percent increase is obtained between 25 °C and 45 °C. It means that if a measurement accuracy of about +/- 2% is required, the K-factor and the actual sample measurements should be performed within about a three degree temperature window.

### Limitations of pathlength correction

Pathlength correction is based on the absorption of water in the near IR area. It can therefore be used only with samples in a water-based buffer, or at least a buffer that has a significant water content. A typical liquid pathlength in a microplate assay can be assumed to be around 2–5 mm, corresponding roughly to a 100–200  $\mu$ l assay volume in 96-well plates, and 25–60  $\mu$ l in 384-well plates. This means that the expected absorbance at 975 nm is about 0.03–0.09 with pure water. When the lowest absorbance level that can be measured reliably is ~0.02 Abs, at least a 60% water content is required for sufficient water absorption at 975 nm.

Another limitation is that there must not be any other assay component than water that absorbs light in the 900–975 nm area. In addition, pathlength correction cannot be used in turbidometric assays where light scattering from solid particles is measured. The particles cause some scattering in the near IR area and therefore the pathlength correction based on water absorption at 900 and 975 nm is not applicable.

### Determining the K-factor

When water or a weak aqueous buffer is used in the assay, you can use a pre-determined value of 0.173 as the K-factor in pathlength correction measurements.

If you want to measure the K-factor yourself, add assay buffer to a cuvette and measure the absorbance both at 975 nm and 900 nm using any photometer capable of measuring in the IR area. Then perform a subtraction  $A_{975\text{ nm}} - A_{900\text{ nm}}$ . That value is the K-factor of the buffer.

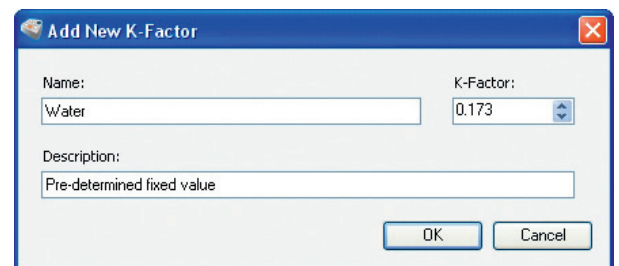
The K-factor should be measured with a polyacrylic plastic UV-cuvette with a 10 mm optical pathlength. A quartz cuvette should not be used unless the microplate assay is also done using a quartz bottom plate. The quartz and plastics have slightly different behaviour in the IR area.

### Setting up a DNA assay with SkanIt Software for Multiskan GO

SkanIt Software is able to perform the pathlength correction automatically by using the K-factor entered in the software. To perform DNA assays on microplates with pathlength correction, follow the procedure below:

#### I) Defining the K-factor in SkanIt Software

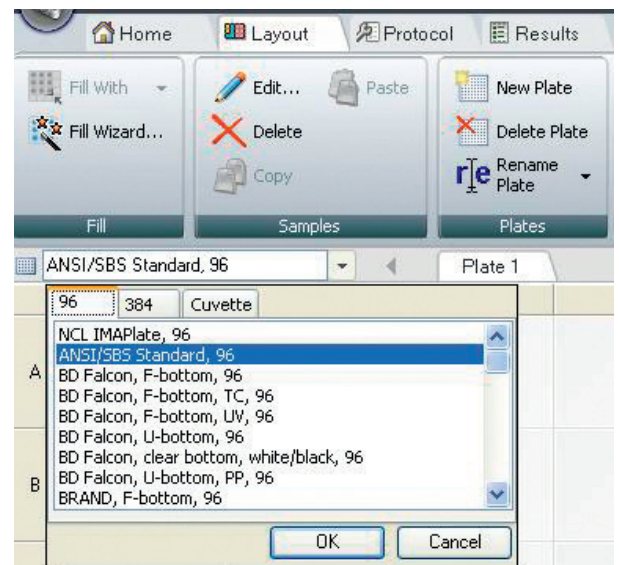
1. Select Home > Settings > K-factors.
2. Select the microplate that you are going to use in the measurements and click “Add K-factor”.
3. Type the buffer name and enter the predetermined value of 0.173 for water and aqueous buffers (or enter the value you have measured yourself).
4. Click OK.



The K-factor is now saved in SkanIt Software and it can be used for any pathlength correction assay using water or aqueous buffers.

#### II) Defining the DNA measurement protocol

1. Create a new measurement protocol by clicking New in the Home view.
2. Select the correct 96- or 384-well plate template in the Layout view. (Note: select the same plate template for which you entered the K-factor).

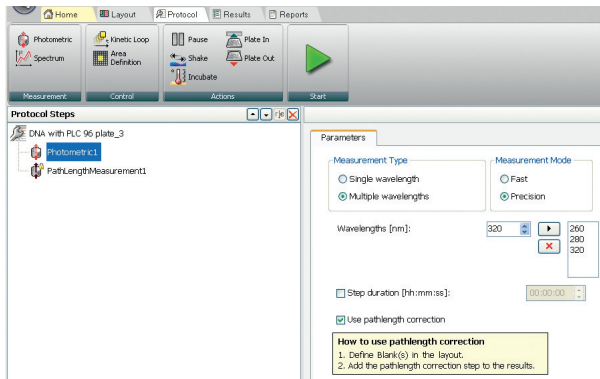




3. Define the samples in the Layout view. Note that at least one blank well is required for the plastic background subtraction.

4. Add the measurement step in the Protocol view. Select a multi-wavelength measurement with all the required wavelengths (for example, 260 nm, 280 nm and 320 nm).

5. Select “Use pathlength correction” to activate the automatic pathlength correction measurements.



6. Save the protocol.

### III) Measuring the DNA assay plate

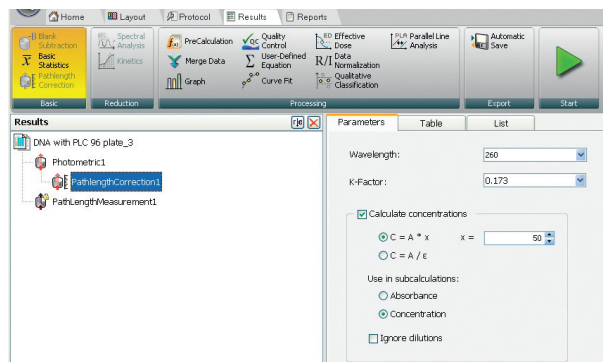
Add the DNA samples and blanks to a UV-quality microplate. For 96-well plates at least 100 µl/well, for 96-well half area plates ~40 µl/well and for 384-well plates at least 25 µl/well is required. Place the microplate into the reader and start the measurement.

### IV) Calculating the DNA concentrations

1. Add the Pathlength Correction calculation step to the DNA data step (Photometric1) and select 260 nm as the data source. Pathlength correction calculation step will automatically perform blank subtraction, therefore it is not necessary to add blank subtraction step prior to pathlength correction calculation step

2. Select the K-factor to be used for pathlength correction.

3. Select “Calculate concentrations” and enter the concentration factor (x) with which the corrected absorbance values should be multiplied (e.g., x=50 for dsDNA).

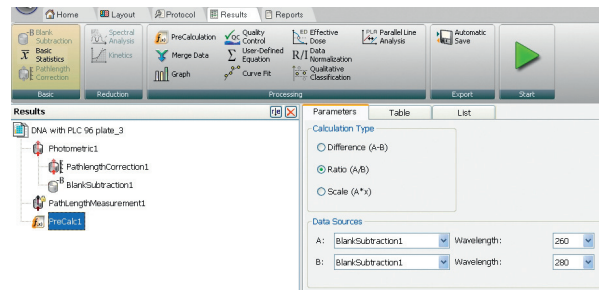


By clicking the “Table” tab you can see the DNA concentration results as a table, and by clicking the “List” tab you can see all the data as a list.

### V) Calculating the 260/280 ratio for purity analysis

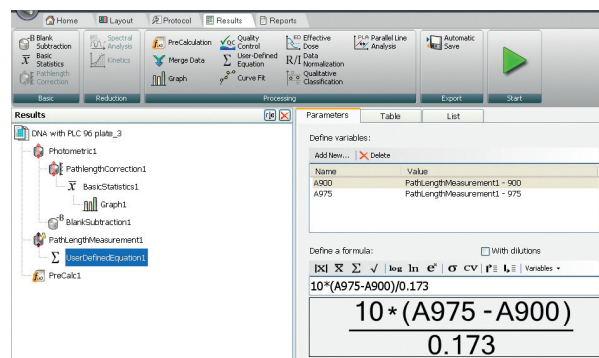
1. Add the Blank Subtraction substep to the Photometric1 step.

2. Add the Precalculation step and select the Ratio (A/B) as the calculation type, Blank Subtraction as the source data and wavelengths A = 260 and B = 280.



### VI) Calculating the pathlength of each well

If you are interested in using the individual liquid pathlengths of each well, you can calculate those values from the 975 and 900 nm microplate data according to Function 3 above by using the User-defined Equation feature of SkanIt Software.



### Example of the DNA assay results using pathlength correction

The performance of the pathlength correction was evaluated by measuring a DNA standard series of known concentrations (in TE buffer). Different volumes per well were used: 100  $\mu$ l, 200  $\mu$ l or 300  $\mu$ l with a 96-well plate,

and 20  $\mu$ l, 40  $\mu$ l or 80  $\mu$ l with a 384-well plate. The plates were measured with the Multiskan GO microplate spectrophotometer and the pathlength correction was performed by SkanIt Software. The results of the 96-well plate are shown in Figure 7 and the results of the 384-well plate are shown in Figure 8.

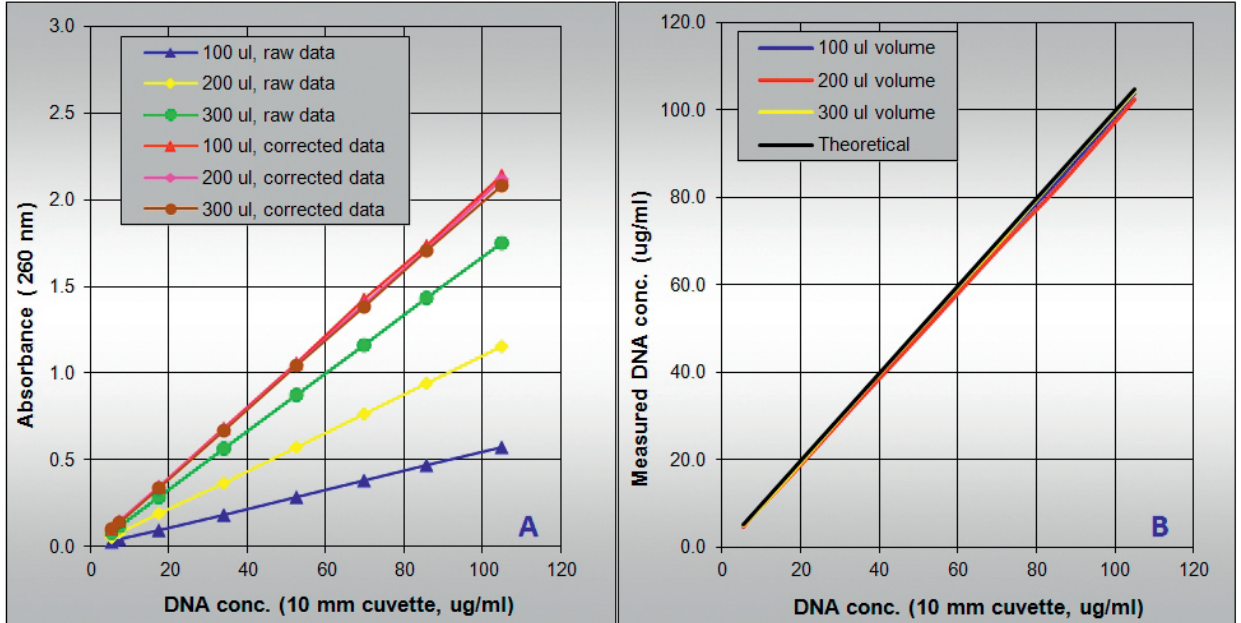


Figure 7. DNA standard curves on a 96-well plate using A<sub>260</sub> nm principle and pathlength correction. A) Raw data absorbance and pathlength corrected absorbance of different assay volumes.

B) DNA concentrations calculated from the pathlength corrected absorbance.

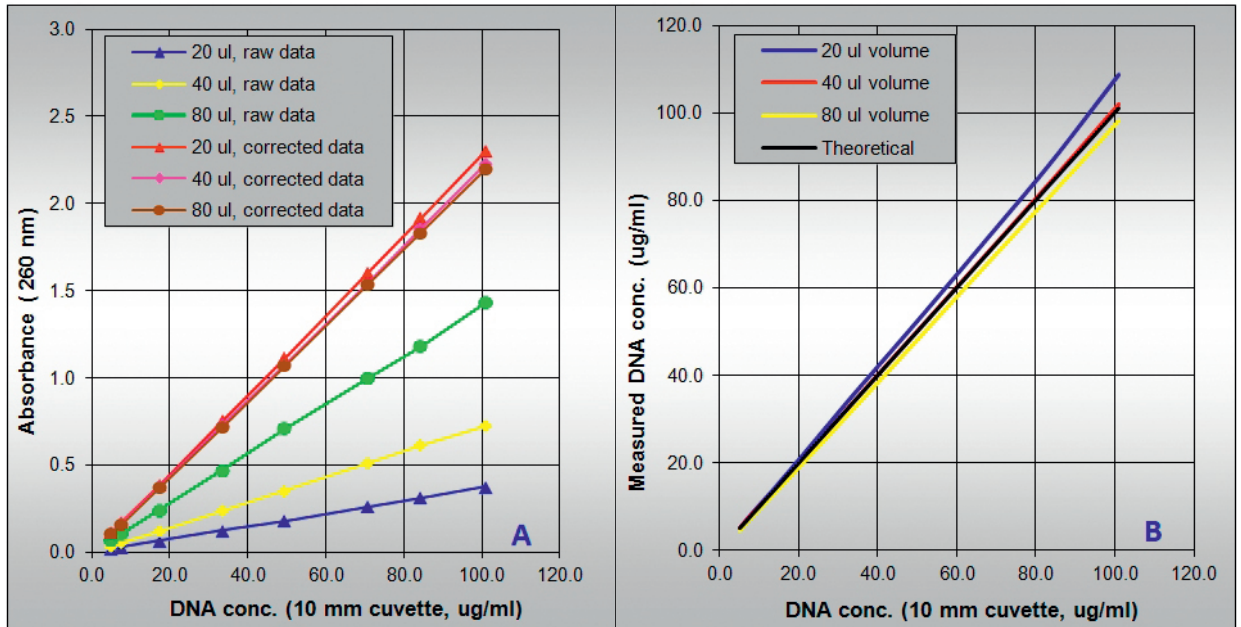


Figure 8. DNA standard curves on a 384-well plate using A<sub>260</sub> nm principle and pathlength correction. A) Raw data absorbance and pathlength corrected absorbance of different assay volumes.

B) DNA concentrations calculated from the pathlength corrected absorbance.

As seen in Figures 7 and 8, the raw absorbance clearly depends on the assay volume, but after pathlength correction, volume independent results are obtained. The microplate results after pathlength correction correlate perfectly with the cuvette results. The only exception seen in the figures is a slight variation between the 20  $\mu$ l assay volume results using 384-well plates and the cuvette results. The volume is so low that the absorption starts to be influenced by the measurement accuracy and precision of the reader causing cumulative error in the pathlength correction mathematics.

A summary of the characteristic DNA assay performance values is given in Table I. The Limit of Detection is around 0.3–0.5  $\mu$ g/ml of DNA with a 384-well plate and between 0.5–0.9  $\mu$ g/ml with a 96-well plate. This is the lowest concentration that can be reliably separated from the blank level, so one can say whether a sample contains DNA or not. For reliable numeric concentration value one has to have at least the concentrations defined by the Limit of Quantification (the lowest limit that can be quantified reliably) which is between 1–2  $\mu$ g/ml with 96-well plates and little below 1  $\mu$ g/ml with 384-well plates. That same inaccuracy already seen in Figure 8 with a 20  $\mu$ l volume also affects the calculated quantification limit that shows to be unexpectedly high, around 3  $\mu$ g/ml.

Photometric DNA assay performance summary		
Assay volume ( $\mu$ l)	96-well plate	
	Limit of Detection ( $\mu$ g/ml)	Limit of Quantification ( $\mu$ g/ml)
100	0.9	2.1
200	0.9	1.7
300	0.5	0.9
Assay volume ( $\mu$ l)	384-well plate	
	Limit of Detection ( $\mu$ g/ml)	Limit of Quantification ( $\mu$ g/ml)
20	0.5	3.1
40	0.3	0.7
80	0.3	0.5

Table I. The performance of photometric DNA assay with different microplate formats.

Variations in the optical pathlengths between the wells, possibly caused by sample differences, can be evaluated by calculating the individual pathlengths based on the pathlength correction data. Figure 9 shows an example of the results of such a calculation.

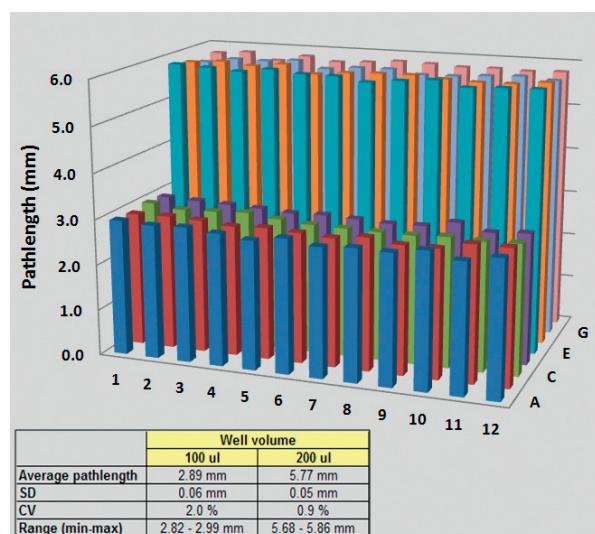


Figure 9. An example of the pathlength calculation based on  $A_{975}$  nm –  $A_{900}$  nm difference. Two volumes, 100 and 200  $\mu$ l, were used on this plate and these volumes produced 2.9 and 5.8 mm optical pathlengths on this UV-quality 96-well plate.

## Conclusions

Photometric pathlength correction is a reliable method for normalizing the microplate results to correspond with the data measured with a cuvette with a 10 mm pathlength. It also makes it possible to use pre-determined calculation factors or molar extinction coefficients for concentration calculations without a standard curve. Therefore the Multiskan GO spectrophotometer with SkanIt Software is an ideal tool for DNA/RNA analysis, in addition to many other photometric applications. This same pathlength correction principle applies also to the Thermo Scientific Varioskan Flash multimode reader.

When the assay is performed in a weak aqueous buffer it is possible to use a pre-determined K-factor of 0.173 for pathlength correction. It makes the procedure easy, straightforward and reliable.

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