Technical Notes

Microplate-Based Pathlength Correction Method for Photometric DNA Quantification

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Abstract

This technical note describes the theory and performance of a photometric pathlength correction method available in the Thermo Scientific™ Multiskan™ GO spectrophotometer or Thermo Scientific™ Varioskan™ LUX multimode reader.

Pathlength correction normalizes absorbance values measured on a microplate to correspond with absorbance values measured in a standard cuvette. The liquid pathlength in a standard cuvette is 1 cm, whereas the liquid pathlength on 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.



Theory

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

$$A = \varepsilon * C * d$$

Where:

A = Absorbance

ε = Extinction coefficient (molar absorption coefficient)

C = Concentration

d = Pathlength (in cm)

Function 1. The Beer-Lambert Law

When A, ε and d (normally 1.0 cm) are known, the concentration can be calculated as:

$$C = \frac{A}{\varepsilon * d}$$

Function 2.

When measuring, e.g., 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 the Beer-Lambert Law can be applied to microplate measurements. If the microplate assay includes standards with known concentrations, pathlength correction is not needed.

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Factors affecting the liquid pathlength on a microplate

The liquid pathlength on microplate wells is mainly dependent 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 be pipetted into the well. The microplate wells have a conical shape, which complicates pathlength calculation.

These factors influence the liquid pathlength:

- Liquid volume
- Plate type and manufacturer
- Buffer composition
- Nucleic acid concentration
- Temperature of the solution

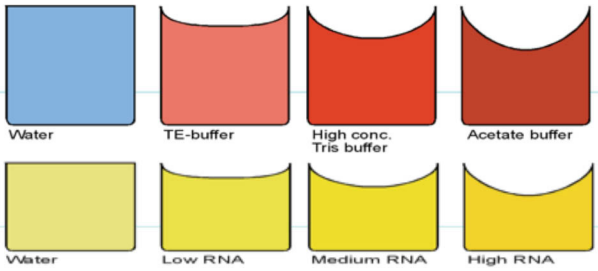


Figure 1. Different solutions can have different meniscus shape and therefore different pathlengths with exactly the same volume.

Determining the liquid pathlength

The liquid pathlength on a microplate well can be measured from water absorption at a near-infrared (near-IR) wavelength area. Figure 2 shows the absorption spectrum of water measured with either a 10 mm polyacrylic disposable cuvette or a 10 mm quartz cuvette. The absorption maximum is at 975 nm, which is the most optimal wavelength for measuring liquid pathlength. A reference measurement at 900 nm eliminates the background absorbance of 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 of a microplate can then be calculated by Function 3.

$$\text{Pathlength} = \frac{\text{A975 (well)} - \text{A900 (well)}}{\text{A975 (cuvette)} - \text{A900 (cuvette)}} * 10\text{ mm} = \frac{\text{A975 (well)} - \text{A900 (well)}}{\text{K-Factor}} * 10\text{ mm}$$

Function 3. Pathlength calculation

When the actual liquid pathlength has been determined, the corrected absorbance value corresponding to the 10 mm pathlength can be calculated by Function 4.

$$\text{Acorrected} = \text{Araw} * \frac{10\text{ mm}}{\text{Pathlength (well)}} \quad \text{Araw} * \frac{\text{K-Factor}}{\text{A975 (well)} - \text{A900 (well)}}$$

Function 4. Corrected absorbance value calculation

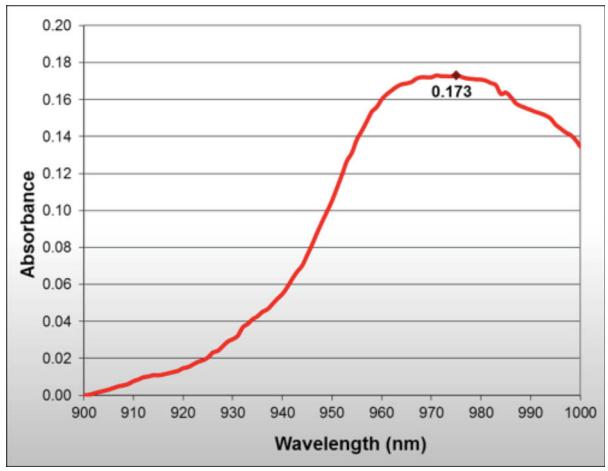


Figure 2. Absorption spectrum of water at a near-IR wavelength area. The raw spectra was subtracted with the reference background value measured at 900 nm. Absorption maximum is at 975 nm. Absorption maximum value 0.173 at 975 nm is the pathlength correction K-Factor for pure water.

Limitations of pathlength correction

Pathlength correction is based on the absorption of water at a near-IR wavelength area. Therefore, it can be used only with samples in a water-based buffer, or a buffer that has at least 60% water content.

Another limitation is that other than water, there must not be any other assay component that absorbs light at 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 at the near-IR area and therefore, the pathlength correction based on water absorption at 900 and 975 nm is not applicable.

Effect of the buffer composition and temperature

The K-Factor is dependent on the composition of the assay buffer. Therefore, the K-Factor should be measured with a buffer composition as close as possible to the solution that is going to be used in the actual microplate assays. 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. (See Figure 3)

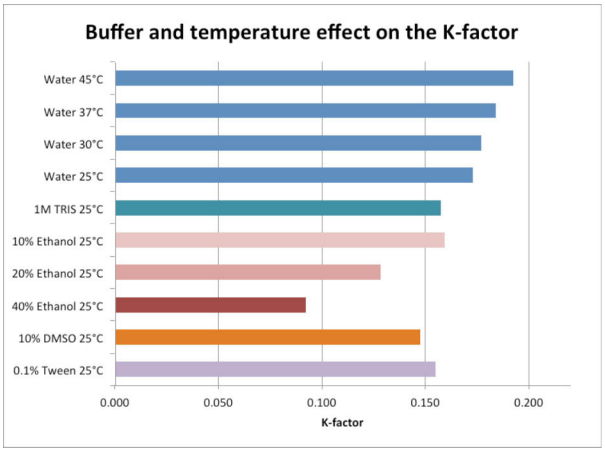


Figure 3. Pathlength correction K-Factors of different buffer compositions and temperature

The fourth generation Thermo Scientific™ SkanIt™ software for microplate readers includes pre-determined K-Factors for commonly used solutions.

Example of the DNA assay results using pathlength correction

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 µl, 200 µl or 300 µl with a 96-well plate and 20 µl, 40 µl or 80 µl with a 384-well plate. The plates were measured with a Multiskan GO microplate spectrophotometer, and the pathlength correction was performed by SkanIt Software. The results of the 96-well plate are shown in Figure 4 and the results of the 384-well plate are shown in Figure 5.

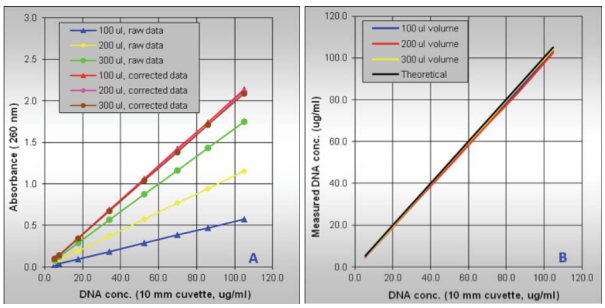


Figure 4. DNA standard curves on a 96-well plate using A260 nm principle and pathlength correction. Graph A shows the raw data absorbance and pathlength corrected absorbance of different assay volumes. Graph B shows the DNA concentrations calculated from the pathlength corrected absorbance.

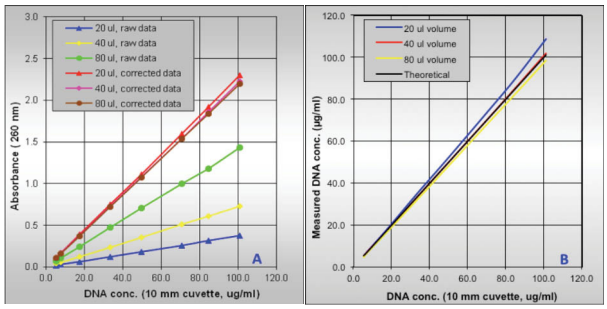


Figure 5. DNA standard curves on a 384-well plate using A260 nm principle and pathlength correction. Graph A shows the raw data absorbance and pathlength corrected absorbance of different assay volumes. Graph B shows the DNA concentrations calculated from the pathlength corrected absorbance.

As seen in Figures 4 and 5, the raw absorbance is clearly dependent on the assay volume, but after pathlength correction is performed, the results are no longer dependent on the volume. 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 µl 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, and this causes cumulative errors in the pathlength correction mathematics.

Summary of the characteristic DNA assay performance values is shown in Table I. The limit of detection is around 0.3–0.5 µg/ml of DNA with a 384-well plate, and between 0.5–0.9 µg/ml with a 96-well plate. This is the lowest concentration that can be reliably separated from the blank level in order to determine whether a sample contains DNA or not. For reliable numeric concentration values one must have at least the concentrations defined by the limit of quantification—the lowest limit that can be reliably quantified—which is between 1–2 µg/ml with 96-well plates, and a little below 1 µg/ml with 384-well plates. The same inaccuracy already seen in Figure 5 with a 20 µl volume also affects the calculated quantification limit that shows to be unexpectedly high – around 3 µg/ml.

Photometric DNA assay performance summary		
Assay volume (µl)	96-well plate	
	Limit of detection (µg/ml)	Limit of quantification (µg/ml)
100	0.9	2.1
200	0.9	1.7
300	0.5	0.9
Assay volume (µl)	384-well plate	
	Limit of detection (µg/ml)	Limit of quantification (µg/ml)
20	0.5	3.1
40	0.3	0.7
80	0.3	0.5

Table 1. The performance of photometric DNA assay with different microplate formats.

Conclusions

Photometric pathlength correction is a reliable method for normalizing the microplate results to correspond with data measured using a cuvette of a 10 mm pathlength.

Pathlength correction enables the use of pre-determined calculation factors—or molar extinction coefficients—for concentration calculations without a standard curve.

With a weak aqueous assay buffer use a pre-determined K-Factor of 0.173 for pathlength correction. It makes the procedure easy, straightforward and reliable.

Multiskan GO spectrophotometer or Varioskan LUX multimode readers with the SkanIt software are excellent tools for DNA/RNA analysis, in addition to many other applications.

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