APPLICATION NOTE

Optical density measurements automatically corrected to a 1-cm pathlength with PathCheck Technology

Introduction

UV/VIS spectrophotometers and microplate readers differ fundamentally in their beam geometry. In spectrophotometers, samples are read through cuvettes or tubes with a horizontal (cross-sectional) light path. The horizontal light beam and the customary 1-cm pathlength make assays based on extinction coefficients straightforward and allow easy comparison of results between labs. In microplate readers, the vertical light beam results in a pathlength that depends on the volume of fluid in each well.

The variable pathlength in microplates has made it difficult to perform extinction-based assays and confusing to compare results obtained in a microplate reader with those obtained in a spectrophotometer. This problem has been remedied by the introduction of Molecular Devices PathCheck® Technology, which enables determination of the pathlength in each well of a microplate and automatically normalizes the absorbance value to a 1-cm pathlength. This application note discusses the principles on which PathCheck Technology is based, and gives specific instructions for using it with SpectraMax® microplate readers and SoftMax Pro Software.

Principles of PathCheck

Pathlength correction using near infrared measurements

Water is essentially transparent from 200 nm to 900 nm, but in the near infrared (NIR), it has a distinctive absorption peak near 977 nm (Figure 1). This characteristic absorbance band, located in a spectral region where most biological molecules have little or no absorbance, can be utilized to measure the pathlength of light through an aqueous sample.

Lambert's law of light absorption predicts absorbance is proportional to the distance that light travels through the sample: the longer the pathlength, the higher the absorbance peak. Baseline absorbance can be measured at a wavelength distant from the water peak, e.g. 900 nm. The pathlength of light through an aqueous sample can be calculated by comparing the peak height obtained in a microplate well with the peak height obtained in a standard 1 cm cuvette:

$$\frac{(A_{280})\ \text{sample} \times (A_{1000}-A_{900})\ \text{1.0 cm aqueous solvent}}{(A_{1000}-A_{900})\ \text{sample}} = A_{280}\ \text{sample corrected to 1.0 cm}$$

In practice, 977 nm is not an ideal wavelength for pathlength measurements because at that wavelength the absorption of water is temperature-dependent. Pathlength measurements are subject to error if the microplate and cuvette measurements are not made at exactly the same temperature. SpectraMax microplate readers avoid temperature-dependency by measuring water absorbance at 1000 nm, near a temperature isosbestic point.

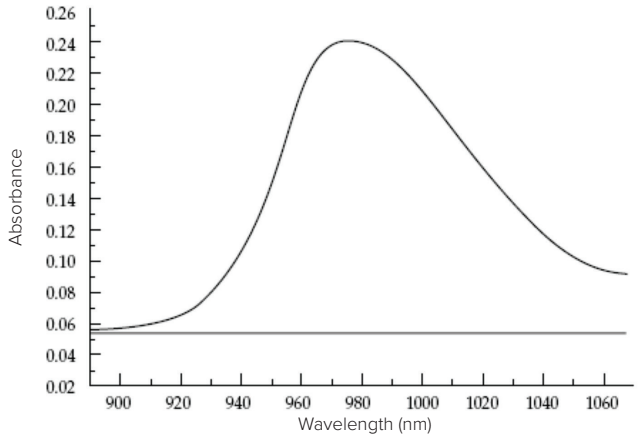

Figure 1. Absorbance spectrum of water. The absorbance spectrum of water is shown, with absorbance peak at 977 nm.

Benefits

- **Automatic correction of variable microplate well volumes for more accurate results**
- **Absorbance-based quantitation without standard curves**
- **Temperature-independent pathlength correction**

The trade-off is an increased requirement for the instrument to have superior wavelength reproducibility, because the measurement is made on a slope of the absorbance curve, rather than at the absorbance maximum. Using the ratio in the equation above, the absorbance of the aqueous sample in a microplate well (in the example below, protein at 280 nm) can be "corrected" to a 1-cm pathlength as follows:

$$\frac{(A_{280})\ \text{sample} \times (A_{1000}\text{-}A_{900})\ \text{1.0 cm aqueous solvent}}{(A_{1000}\text{-}A_{900})\ \text{sample}} = A_{280}\ \text{sample corrected to 1.0 cm}$$

The pre-installed Water Constant: a shortcut

Because comparatively few substances absorb in the NIR, the quantity $(A\ 1000 - A900)_{1.0\ cm\ aqueous\ solvent}$ in the equation above is, for aqueous solutions, practically a constant, and is referred to as the "Water Constant". The value of the Water Constant for each SpectraMax microplate reader is determined during instrument manufacture and is pre-installed into the instrument's firmware. Substituting the Water Constant for $(A1000 - A900)_{1.0\ cm\ aqueous\ solvent}$ in the equation for correcting absorbance values in the UV/VIS region then yields:

$$\frac{(A_{280})\ \text{sample} \times \text{Water Constant}}{(A_{1000}\text{-}A_{900})\ \text{sample}} = A_{280}\ \text{sample corrected to 1.0 cm}$$

Pathlength correction by SpectraMax microplate readers

Two ways to correct pathlength

SpectraMax microplate readers give you two options when making pathlength-corrected measurements: 1) putting the aqueous solvent into a clean 1-cm reference cuvette in the instrument's cuvette port and having the instrument base the calculations on the A1000 and A900 of the cuvette (available only for readers with a cuvette port), or 2) using the Water Constant. A cuvette reference is only necessary in cases where the sample solvent is greater than 0.5 M in salts or solutes, contains organic solvent or has unusual absorbance in the NIR. Use of the Water Constant is potentially less accurate.

In addition to reporting absorbance measurements "corrected" to a 1-cm pathlength, SoftMax Pro Software can report the pathlength in each of the 96 wells. This ability is useful to screen a microplate for volume irregularities or to check for pipetting errors. When performing an absorbance read with PathCheck, SoftMax Pro Software performs the following sequence of calculations:

1. Subtraction of the Plate Background OD (if selected in Data Reduction).
2. Pathlength correction (using the ratio of the NIR measurements).
3. Subtraction of plate/group blanks (if any).
4. Additional user-specified custom data reduction (if any).

Using PathCheck

Getting ready

Materials

1. SpectraMax microplate reader
2. High-quality UV-clear microplates, e.g. quartz or UV-transparent polymer
3. Pipettor and tips or transfer pipets suitable for use with microplates
4. Samples (100-300 µL each)

Set up SoftMax Pro Software to use PathCheck

1. Launch SoftMax Pro Software, then open a Plate Section or, if necessary, create a new Plate Section.
2. Set up the Instrument Settings as indicated in Table 1. Select to perform an endpoint read at the desired wavelength(s). Click the PathCheck box to mark it, then click the Water Constant or Cuvette Reference button, depending upon which you wish to use. Note: Cuvette Reference is only available when using an instrument with a cuvette port.
3. Use the Template Editor to create a template showing where standards and unknowns will be located in the microplate.
4. Click the Data Reduction button in the toolbar to display the Data Reduction dialog box (Figure 2). Check the box next to 'Apply PathCheck'. If a Plate Background OD will be used (must be used if wells do not contain identical volumes), check the box next to 'Apply Plate Background OD' and enter a value. (See 'Determination of Plate Background OD below for instructions.) Set the Raw Data Mode to Optical Density. Set the Wavelength Options, for example '!Lm1' for single wavelength reads.

Determination of Plate Background OD

Pipet 100-200 µL of deionized water into each well of a clean 96-well microplate. Volume is not critical because water does not absorb light appreciably between 190 and 900 nm. Read absorbance on the SpectraMax microplate reader at the wavelength to be used for your assay, and average the resulting OD values for all wells to obtain the Plate Background OD. Enter this value into the Data Reduction dialog box (see Figure 2).

Pathlength correction using a cuvette reference

Note: Cuvette Reference is only available with SpectraMax microplate readers that have a cuvette port.

1. Set up SoftMax Pro Software to use PathCheck as detailed above. Select 'Cuvette Reference' in the Instrument Settings.
2. Place a clean quartz or glass cuvette containing distilled water or your aqueous sample solvent in the reader's cuvette port. Plastic cuvettes are also generally acceptable, but the performance of a particular cuvette should be verified before use in an assay.

Parameter	Setting
Read Mode	ABS (Absorbance)
Read Type	Endpoint
Wavelength Settings	[User-defined]
Plate Type Settings	
Read Area Settings	
PathCheck	Check box and select Water Constant or Cuvette Reference (if available)
Shake Settings	[Optional]
Speed Read	
More Settings	[Select according to the instrument's feature set and desired settings.]

Table 1: Instrument settings for absorbance read with PathCheck.

3. Put your assay plate in the plate reader plate carriage, and then click the Read button in the Plate section.

The cuvette will be read at the same time the plate is read, and its NIR absorbance values used in the PathCheck calculations. Note: To avoid errors due to evaporation, make the readings within a few minutes of putting the samples in the microplate. If the read must be delayed, cover the plate with an adhesive seal. Remove the seal immediately prior to reading the plate.

Pathlength correction using the Water Constant

Note: for SpectraMax microplate readers without a cuvette port, the water constant is automatically used in PathCheck calculations, and no Water Constant option appears in the Instrument Settings.

1. Set up SoftMax Pro Software to use PathCheck as detailed above. Select 'Water Constant' in the Instrument Settings dialog box (Figure 3). For readers without a cuvette port, simply check the box next to 'PathCheck'.
2. Put your assay plate in the plate reader plate carriage, and then click the Read button in the Plate section.

Note: To avoid errors due to evaporation, make the readings within a few minutes of putting the samples in the microplate. If the read must be delayed, cover the plate with an adhesive seal. Remove the seal immediately prior to reading the plate.

Using PathCheck to inspect a microplate for pipetting errors

1. In SoftMax Pro Software, select a Plate Section or, if necessary, create a new Plate by selecting New Plate from the Experiment menu.
2. Set up the Instrument Settings as indicated in Table 1. Select to perform an endpoint read at any wavelength. Note: SoftMax Pro Software requires that you enter a wavelength at which to read, even though that measurement is not used in calculating pathlength. Select PathCheck, with Water Constant or Cuvette Reference if available.
3. Click the Data Reduction button in the Plate section's tool bar to display the Data Reduction dialog box. Select '!Pathlength' from the Wavelength Options dropdown menu, as shown in Figure 4.

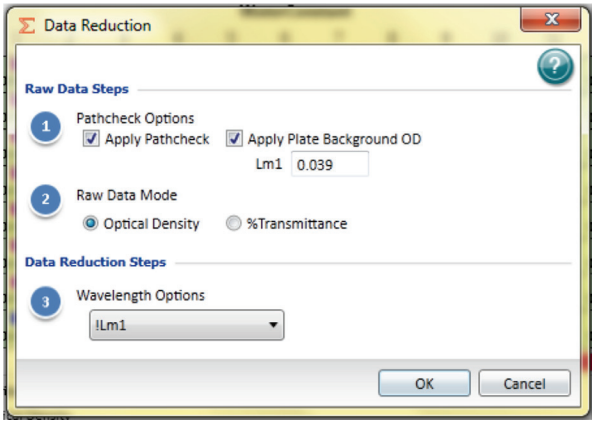

Figure 2. Data Reduction dialog box. Data Reduction settings shown for a typical absorbance read using PathCheck are shown. Plate Background OD must be applied if wells do not contain identical volumes.

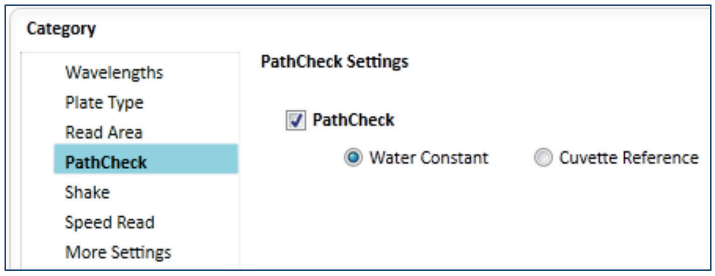

Figure 3. Selection of Water Constant in PathCheck settings. Water Constant and Cuvette Reference options are available on SpectraMax microplate readers with a cuvette port. For readers without a cuvette port, these options are not shown in the software.

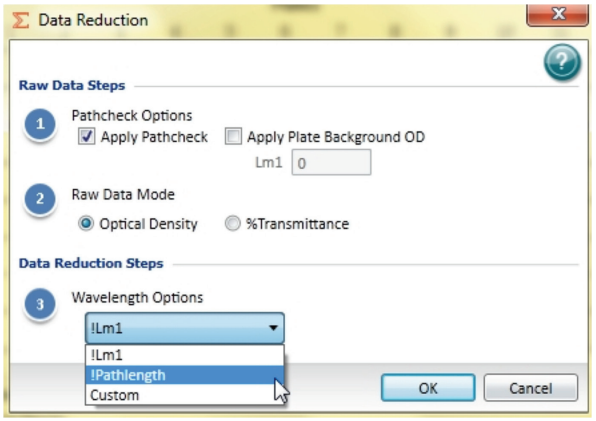

Figure 4. Data Reduction dialog box. When '!Pathlength' is selected from the Wavelength Options dropdown menu, the calculated pathlength for each well will be displayed in the Plate section once the plate is read.

4. For quick visualization of the plate it is useful to display the data using the gray scale or color map option. Click the Display Options button in the Plate section's toolbar to open the Display Options dialog box (Figure 5), then select 'Gray scale' or 'Color map' from the dropdown menu. If desired, you can also select to have the reduced number displayed in each well.
5. Put the plate into the instrument's plate carriage, and then click the Read button.

Experimental Results

Experimental design and cuvette results

A yellow pH 7 buffer solution (e.g. Ricca Chemical Company cat. #1551) was used to illustrate the performance of the SpectraMax microplate reader and PathCheck. The buffer has an absorbance maximum at 426 nm. Buffer was pipetted into wells of a Greiner 96-well clear microplate (cat. #655101) in volumes of 75 to 300 µL, and the absorbance was measured, using PathCheck to normalize it to a 1-cm pathlength.

Note: 75 µL is less than the recommended minimum volume per well, but was included to illustrate the variability occurring at marginal volumes.

A Cuvette Set was created by clicking 'New Cuvette Set' in the toolbar, and in the Cuvette Set Template dialog box, a new group called 'Cuvette' was created, comprising 3 samples named "Buffer" (Figure 6).

In the Settings for the Cuvette Set, an endpoint read at 426 nm was specified. A glass cuvette was filled with deionized water and placed in the cuvette port of the SpectraMax microplate reader. The 'Ref' button in the toolbar was clicked to perform a reference read on the cuvette. The cuvette was then replaced with a matched cuvette containing the yellow buffer, and the absorbance of the yellow buffer was read three times. Figure 7 shows the resulting absorbance values in the Cuvette Set and in the Group Table. The results indicate that the absorbance of the yellow buffer at 426 nm in a standard 1 cm cuvette was 0.556.

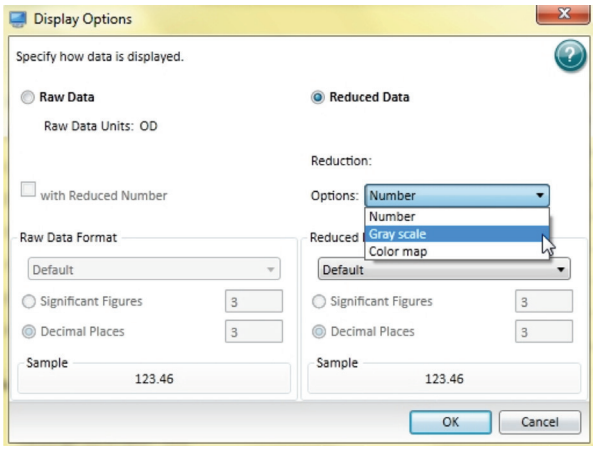

Figure 5. Display Options dialog box. Gray scale and color map options provide a way to quickly visualize the data across an entire microplate.

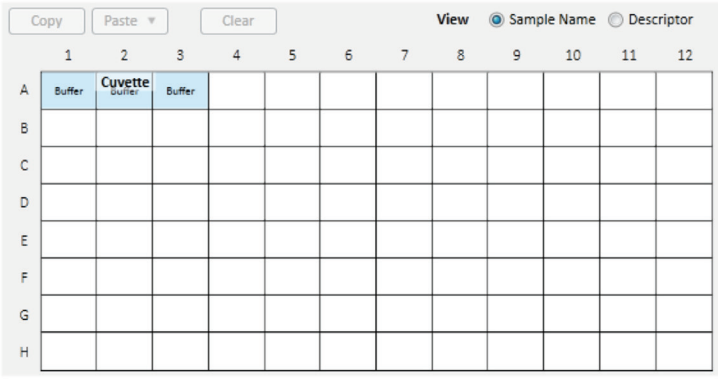

Figure 6. Template editor for a cuvette section. Assigning samples to a template section, here one entitled Cuvette, creates a group table in which data are displayed and additional calculations may be performed.

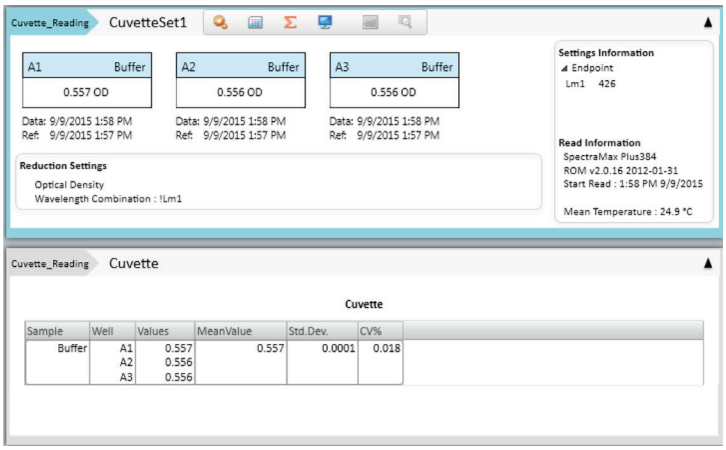

Figure 7. Cuvette set for the yellow buffer, and corresponding 'Cuvette' group table. In the 'Cuvette' group table, mean value, standard deviation, and CV% are calculated automatically. Other group table columns and calculations may be added.

Plate Background OD, which was used for subsequent tests, was measured as follows. 150 µL of deionized water was pipetted into each well of a clean 96-well plate. The absorbance was then read on the SpectraMax microplate reader at 426 nm, and the resulting OD values for all wells were averaged together to obtain a value of 0.039. This value was then entered into the Data Reduction dialog box (see Figure 2).

Yellow buffer was pipetted into a second microplate from the same lot, at volumes ranging from 75 µL to 300 µL per well, as indicated in the template (Figure 8). The cuvette from the previous test was left in the cuvette port, and the plate was read using the cuvette as a reference.

Plate optical densities displayed without pathlength correction

Figure 9 displays the results when PathCheck is de-selected in the Data Reduction dialog box. The plate section is displayed as a color map for easy visualization. As one would predict, without pathlength correction, the absorbance values increase with increasing volume of sample in the microplate wells. Mean OD values calculated in the software range from 0.17 for the 75 µL group to 0.52 for the 300 µL group.

Plate optical densities displayed with pathlength correction

Figure 10 shows the same set of data with PathCheck applied to it. The plate section is shown as a color map with pathlength-corrected OD values also displayed. For all the sample volumes, the corrected values are close to the cuvette value.

Plate data displayed as absolute pathlength in centimeters

Figure 12 shows the same data set displayed in absolute pathlength. Not surprisingly, the color map representation of the data looks similar to that in Figure 10, which displays the raw OD values. Calculated mean pathlength values ranged from 0.23 cm to 0.88 cm.

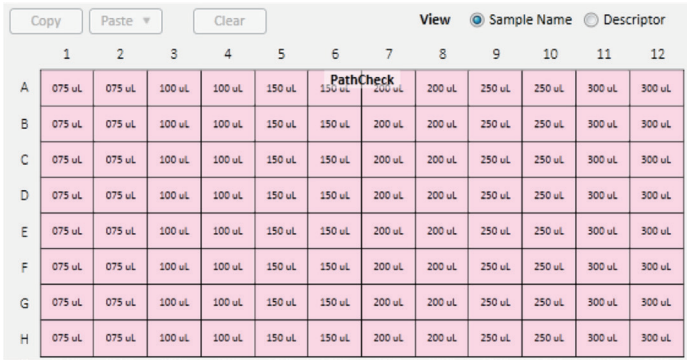

Figure 8. Template showing location of samples of different volumes in the microplate. In the Template Editor window, wells were assigned to a group named 'PathCheck', and names corresponding to the sample volumes were assigned to the wells.

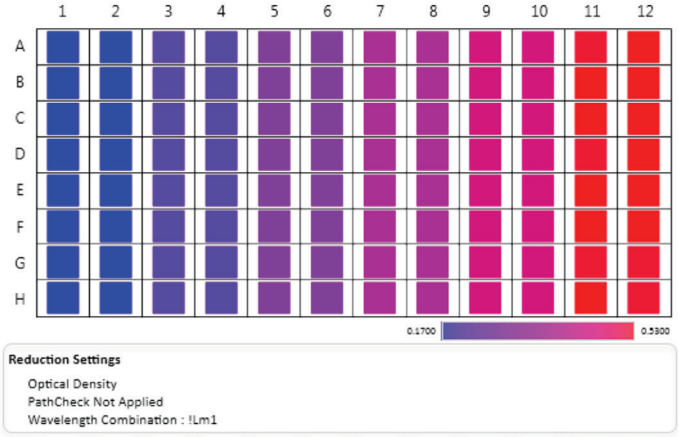

Figure 9. Plate section without PathCheck applied. A plate containing two columns each of 75 µL, 100 µL, 150 µL, 200 µL, 250 µL, or 300 µL of yellow buffer solution was read at 426 nm on a SpectraMax microplate reader. The resulting OD_{426} values are visually represented above as a color map generated in the plate section of SoftMax® Pro Software. Raw, uncorrected OD_{426} values ranged from 0.17 to 0.53.

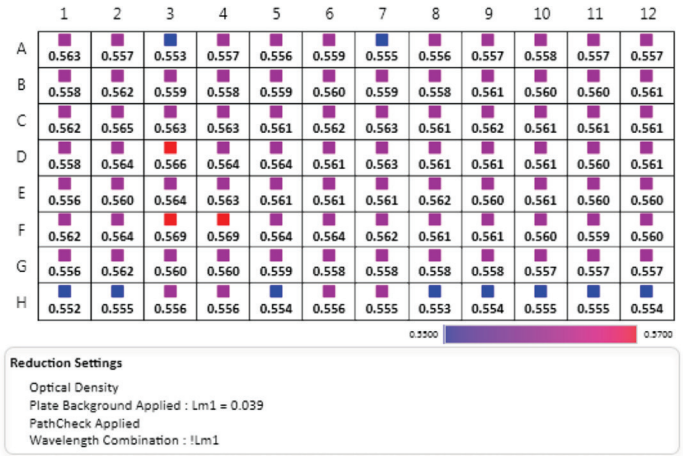

Figure 10. Plate section with PathCheck applied. OD_{426} values were corrected to 1-cm values using PathCheck Technology. Corrected OD_{426} values range from 0.55 to 0.57 and are within 0.5% of the OD_{426} obtained from a cuvette reading of the same solution.

Data combined into a single graph

In Figure 12, absorbance at 426 nm with and without pathlength correction is plotted as a function of pathlength in centimeters. As expected, the uncorrected absorbance values increase with increasing pathlength (i.e., with increasing volume in the wells). When PathCheck is applied, the corrected absorbance values are almost identical.

Discussion

Advice and precautions

When pathlength-corrected measurements are made using an appropriate procedure, the values should be within 2.5% or better of values obtained from the same solution in a 1 cm cuvette. Two potential causes of erroneous results are evaporation and failure to apply a Plate Background OD.

Evaporation

Recall the equation for pathlength correction:

$$\frac{(A_{280})\ \text{sample} \times (A_{1000}\text{-}A_{900})\ 1.0\ \text{cm aqueous solvent}}{(A_{1000}\text{-}A_{900})\ \text{sample}} = A_{280}\ \text{sample corrected to 1.0 cm}$$

If evaporation occurs, the value for $(A_{1000} - A_{900})_{sample}$ in the denominator decreases, causing a progressive overcorrection. (The reading in the UV/VIS region does not change unless the analyte happens to be volatile.) The effect is particularly noticeable in wells with small volumes. Even if the wells contain 300 µL of solution, the effect of evaporation is noticeable within 15 min (~1% error). To avoid errors due to evaporation, take the readings within minutes of putting the samples in the microplate. If the read must be delayed, cover the plate with an adhesive seal, and then remove the seal immediately before reading the plate.

Failure to perform appropriate background subtraction

When using PathCheck to correct for pathlength through a sample, SoftMax Pro Software goes through the following sequence of calculations:

1. Subtraction of the Plate Background OD from the raw absorbance values.
2. Pathlength correction (using the ratio of the NIR measurements).
3. Subtraction of plate/group blanks (if any).
4. Additional user-specified custom data reduction (if any).

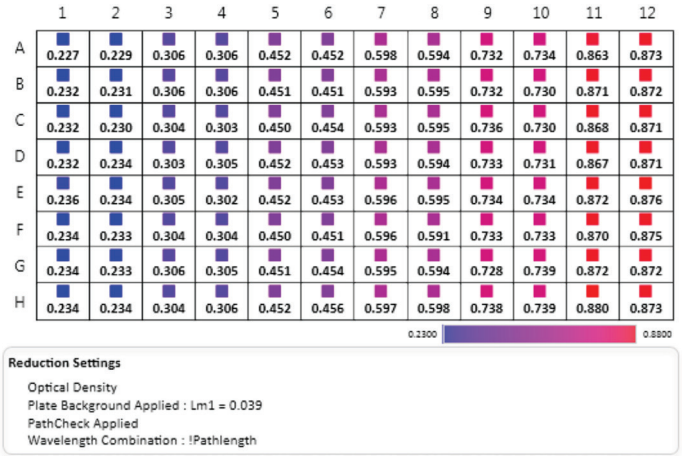

Figure 11. Plate section showing pathlength in centimeters. PathCheck Technology was used to calculate the pathlength for each well, which is displayed in the plate section above and visually represented using a color map.

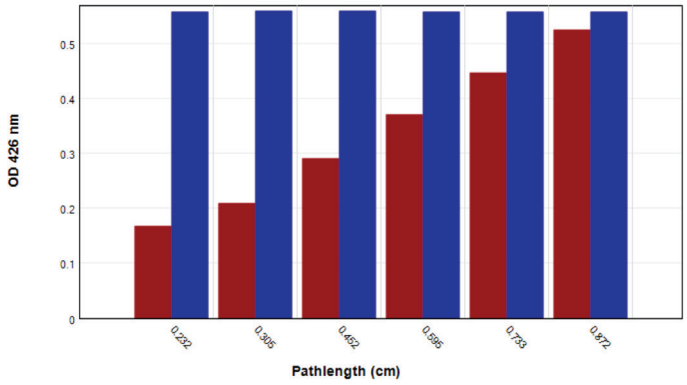

Figure 12. OD_{426} without and with pathlength correction. Red bars, OD values without pathlength correction; blue bars, pathlength-corrected OD values. Average pathlength for samples ranging from 75 to 300 µL are displayed on the y axis.

The purpose of the Plate Background OD is not necessarily to correct for well-to-well variability in the microplate, but instead to subtract the absorbance due to the plate material before pathlength correction is applied. To calculate Plate Background OD, the plate should be read with water in the wells. Wells read with water generally have less absorbance than dry wells. Thus using a dry plate to obtain a Plate Background OD results in pathlength-corrected absorbance values that are lower than the cuvette values. If the Plate Background OD is omitted entirely, the pathlength-corrected results are grossly high.

Potential interference

Pathlength correction is intended for use with aqueous solutions, although small quantities of organic solvents can be tolerated if the PathCheck measurements are made with a cuvette reference. As stated above, a cuvette reference is strongly recommended for pathlength-corrected measurements, although the water constant can often be used as a shortcut. However, if the sample solvent contains a high concentration (e.g. > 0.5 M) of salts or other solutes, or contains organic solvent, the NIR absorbance values may differ from water. In such cases the Water Constant is not appropriate, and it is essential to take pathlength-corrected measurements using a cuvette reference containing the sample solvent.

Pathlength correction using NIR absorbance is appropriate as long as there is nothing in the sample that interferes with the measurements. Turbidity is one source of interference. Also, any molecule with differential absorption at 1000 and 900 nm will interfere if present in a high enough concentration. Although few biological substances absorb between 900 and 1000 nm, interference may be expected from some highly-conjugated molecules such as the pthalocyanines, chlorophylls, carotenoids, phycobilins, as well as porphyrin-containing or related molecules such a myoglobin, hemoglobin and peroxidases. Reduced phosphomolybdate complexes also absorb between 900 and 1000 nm. Pathlength correction in such solutions, however, can still be applied if the interfering substances are dilute enough such their NIR absorption is insignificant relative to water. Provided that the interfering species is present at a constant concentration in all samples, the interference also can be eliminated by placing a sample with the interfering species in the reference cuvette.

Conclusion

PathCheck Technology overcomes the problem of variable pathlength in microplates by enabling determination of pathlength in each well of a microplate and automatically normalizing the absorbance value to a 1-cm pathlength. PathCheck utilizes absorbance of water at 1000 nm to ensure results that are independent of temperature. PathCheck can be used for direct absorbance-based quantitation of nucleic acids, protein, and other analytes, as well as detection of pipetting errors for more reliable results.

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