

PathCheck applied to measurement of protein solutions in the SPECTRAmax[®] PLUS microplate spectrophotometer

INTRODUCTION

Protein concentration is commonly estimated by measuring the absorbance at 280 nm and calculating the concentration using the extinction coefficient of the protein. The procedure is feasible because extinction coefficients are typically obtained using standard cuvettes with a fixed 1 cm pathlength. Extinction-based protein assays can also be done in microplates, but they have not been widely used because of the inconvenience of compensating for variable pathlengths of samples in microplate wells. The introduction of the Molecular Devices' SPECTRAmax PLUS microplate spectrophotometer marks the first time that photometric measurements made in a microplate format can be automatically normalized such that the resultant values are identical to those obtained with the corresponding solutions in a standard 1 cm pathlength cuvette. The following application note outlines the use of the pathlength correction feature "Pathcheck", compares absorbance results obtained in cuvettes and microplate, and gives an example where Pathcheck is applied to column chromatographic fractions and the elution profile displayed.

MATERIALS

For details of the principles of pathlength correction, refer to MAXline application note #25, "Optical density measurements automatically normalized to a 1 cm pathlength in the SPECTRAmax PLUS microplate spectrophotometer".

- 1 SPECTRAmax PLUS microplate spectrophotometer
- 2 UV-transparent microplates; e.g.:
 - SPECTRAplate[®] Quartz, Molecular Devices Corp. (catalog # R8024)
 - SPECTRAplate Quartz half-area microplate, Molecular Devices Corp., (catalog # R8028)
 - Quartz microtest 8-well strip, Hellma (catalog # 730.010QS); use with a frame for 8-well strips, E & K Scientific Products (catalog # 564101). *Note: the 8-well strip frame sold by Evergreen Scientific does not fit properly.*
 - Disposable UV Plate, Corning Costar Corp. (catalog # 3635)
 - UV-MAX, (Polyfiltronics, Inc., catalog # VO32SCXP8)

- 3 Pipettor and tips or transfer pipets suitable for use with microplates.
- 4 Samples (100-300 μL each)
- 5 Quartz cuvette

Set up to make pre-read measurements

- Step 1** Launch SOFTmax PRO[®], then open a Plate section or, if necessary, create a new Plate section.
- Step 2** Set up the Instrument Settings dialog box as shown in Figure 1. In the Instrument Settings dialog box, select to perform an endpoint read at 280 nm. Select PathCheck, (Pre-read plate will automatically be checked) and select Cuvette Reference. (Alternatively, you can use the factory-installed Water Constant as a shortcut, though it is potentially less accurate.)

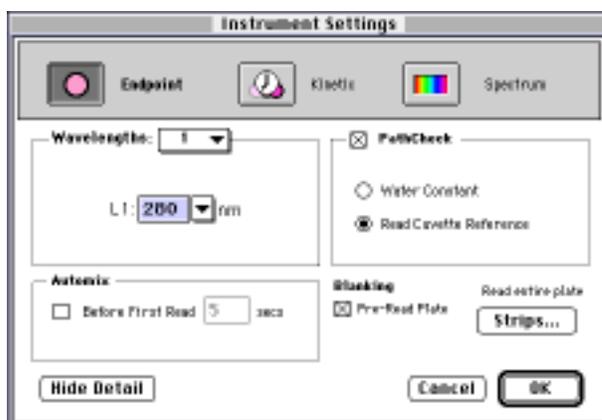


Figure 1: The Instrument Settings dialog box, set up to measure absorbance at 280 nm with PathCheck

- Step 3** Use the Template Editor to create a template showing where standards, blanks, and unknowns will be located on the microplate.
- Step 4** Set up the Reduction dialog box as shown in Figure 2. Set the wavelength combination to L1 and the data mode to Absorbance. Make sure that 'Use pre-read plate' and 'Apply Pathcheck' are selected.

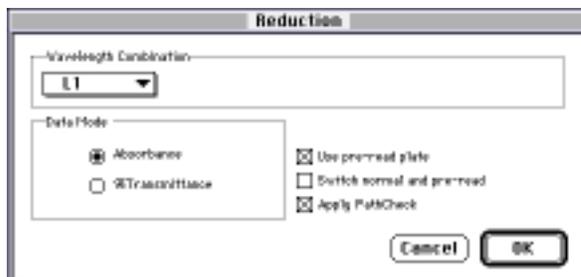


Figure 2: The Reduction dialog box set up to apply PathCheck

METHOD

Making pre-read measurements

The purpose of the pre-read is to subtract the background absorbance of the plate. In contrast, blanks (plate, sample or group) are designated in the template and are subtracted after pathlength correction. In some cases, you may choose to use stored pre-read values (See below).

- Step 1** Select a clean microplate and pipet distilled water into the wells in approximately the same volumes as the intended samples. Accurate pipetting is not necessary.
- Step 2** Put the plate into the drawer of the SPECTRAMax PLUS, then click the Read button in SOFTmax PRO's toolbar to make the pre-read measurements.
- Step 3** Remove the plate, pour out the water and blot the plate dry.

Making pathlength-corrected absorbance measurements

Note: If you have elected to use the pre-programmed Water Constant, you may skip Step 1.

- Step 1** Place a clean quartz cuvette containing your sample buffer or distilled water into the SPECTRAMax PLUS cuvette port.
- Step 2** Transfer aliquots of your samples and blanks into their designated wells in the microplate. Accurate pipetting is not necessary, though the volumes should be between 100 μ L and 300 μ L for best pathlength-corrected results.
- Step 3** Put the plate into the SPECTRAMax PLUS drawer, then click the Read button in SOFTmax PRO's toolbar to make the absorbance measurements. (The cuvette will be read in the near-infrared at the same time the plate is read, and its absorbance values used in the PathCheck calculations.)

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

Storing and re-using pre-read data

Quartz plates are so uniform that it is not necessary to subtract the plate background on a well-by-well basis. Instead, you can pre-read one plate, then store the pre-read data in a SOFTmax PRO file for subsequent re-use, being careful to save the data under a different file name each time, to avoid over-writing the pre-read data.

If you use Costar UV plates, you may or may not want to use stored pre-read values, depending on the precision you require. At 280 nm, the average absorbance of Costar wells containing water is approximately $.050 \pm .002$ OD. Thus, samples having raw absorbance values of 1 OD would be subject to less than 1% potential error due to plate background variation. However, the lower the absorbance in the wells, the greater the maximum potential error due to plate variability.

RESULTS

Example 1: Demonstration of the utility of PathCheck using a protein standard curve

To illustrate the performance of the instrument, a protein “standard curve” was prepared. Bovine serum albumin (BSA) was dissolved in phosphate-buffered saline (PBS), pH 7.4, filter-sterilized and diluted to 6, 5, 4, 3, 2, 1, 0.5 and 0.25 mg/mL. Absorbance measurements made in a microplate were compared with measurements made on the same solutions in a 1 cm cuvette.

SOFTmax PRO was launched and a Group entitled “Plate Standard” was created and given a ‘Standards’ format in the Template Editor of a Plate section. The standards were assigned to wells as illustrated in Figure 3. “Standard 01” was PBS with no added protein (0 mg/mL). An identical template was set up in the Template Editor of a Cuvette Set, except the group was named “Cuvette Standard”.

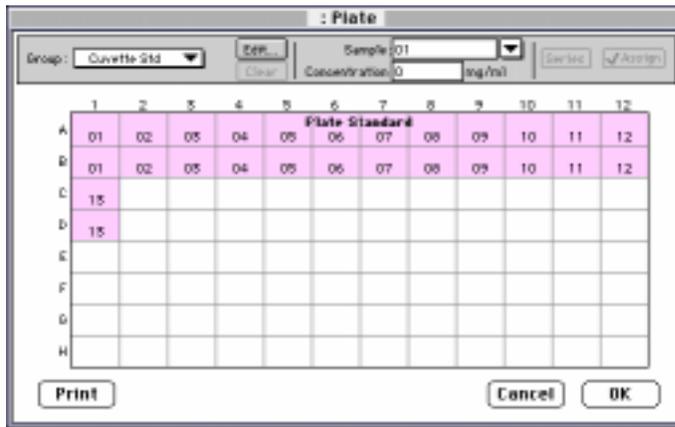


Figure 3: The Template Editor for the Plate section

The instrument settings and data reduction for the Plate section were set up as shown in Figures 1 and 2 above. The CuvetteSet section was set up for an endpoint read at 280 nm.

A cuvette containing PBS was placed in the cuvette port to serve as the cuvette reference for PathCheck. Pre-read measurements were made with 200 μ L water in each well of a Costar UV plate, after which the plate was emptied and blotted dry. Aliquots of the protein ‘standard’ solutions were transferred into their designated wells using disposable transfer pipets. The volumes in each well were deliberately varied in order to demonstrate the utility of the PathCheck feature. The plate was placed into the drawer of the microplate reader and read.

In the Cuvette Set, a reference read was taken on the PBS-containing cuvette in the cuvette port. Then, one by one, each of the protein ‘standard’ solutions was read in the cuvette.

The Group Table associated with the Plate section is shown in Figure 4.

Plate Standard (ng/ml)					
Sample	Concentration	Wells	Values	MeanValue	Raw Values
01	0.000	A1	0.008	0.007	0.004
		B1	0.009		
02	0.125	A2	0.079	0.080	0.097
		B2	0.081		
03	0.250	A3	0.154	0.155	0.099
		B3	0.152		
04	0.500	A4	0.300	0.301	0.170
		B4	0.302		
05	1.000	A5	0.590	0.588	0.397
		B5	0.586		
06	1.500	A6	0.897	0.899	0.609
		B6	0.901		
07	2.000	A7	1.189	1.198	0.707
		B7	1.211		
08	2.500	A8	1.477	1.489	0.841
		B8	1.501		
09	5.000	A9	1.795	1.795	1.255
		B9	1.798		
10	5.500	A10	2.052	2.095	1.248
		B10	2.107		
11	4.000	A11	2.357	2.399	1.097
		B11	2.412		
12	5.000	A12	2.948	2.948	1.671
		B12	2.998		
13	6.000	C1	5.498	5.440	1.958
		D1	5.421		

Figure 4: The Group Table for the Plate section

All columns except the far right column in the Group Table were created automatically by SOFTmax PRO when the "Plate Standard" group was created in the template. The numbers in the "Values" column are absorbance values corrected to a 1.0 cm pathlength. The numbers in the far right column titled "Raw Values" are raw absorbance values (pre-read subtracted) before pathlength correction. These values were obtained by back-calculation using the custom formula shown in the Calculation dialog box for that column (Figure 5). Basically, the Raw Values were calculated by multiplying the corrected values by their corresponding pathlengths and taking the average of the replicates.

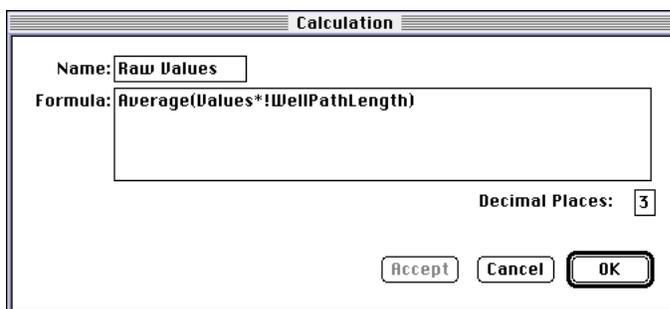


Figure 5: The Calculation dialog box for the "Raw Values" column

The Group Table (“Cuvette Standard”) displays the data from the absorbance readings in the cuvette (Figure 6). The column ‘Values’ contains the absorbance values at 280 nm.

Cuvette Standard (mg/ml)				
Sample	Concentration	Wells	Values	MeanValue
01	0.000	A1	0.004	0.004
		B1	0.004	
02	0.125	A2	0.078	0.078
		B2	0.078	
03	0.250	A3	0.151	0.151
		B3	0.151	
04	0.500	A4	0.300	0.299
		B4	0.299	
05	1.000	A5	0.590	0.589
		B5	0.589	
06	1.500	A6	0.896	0.895
		B6	0.895	
07	2.000	A7	1.195	1.195
		B7	1.195	
08	2.500	A8	1.468	1.470
		B8	1.471	
09	3.000	A9	1.765	1.765
		B9	1.765	
10	3.500	A10	2.007	2.006
		B10	2.009	
11	4.000	A11	2.289	2.258
		B11	2.287	
12	5.000	A12	2.542	2.559
		B12	2.556	
13	6.000	C1	2.685	2.678
		D1	2.675	

Figure 6: The Group Table for the Cuvette Set

Next, the standard curves from the plate and cuvette were plotted in a single graph. A Graph section was created, then the Plots dialog box was used to create three plots by selecting the corresponding Group tables from the Groups pop-up menu, then specifying the x and y variables for each plot (Figure 7). In the example shown in the figure, the highlighted plot is “Plate with PathCheck”. The data came from the group “Plate Standard”, the x variable is concentration, and the y variable is “MeanValue” (i.e. the mean pathlength-corrected absorbance value).

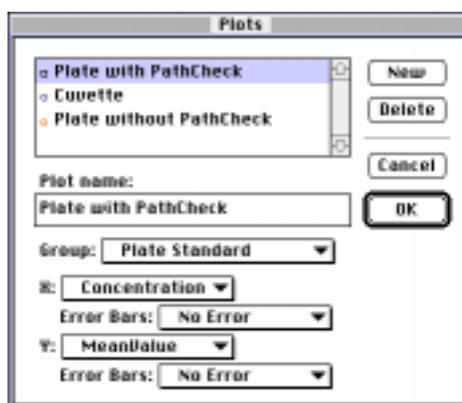


Figure 7: The Plots dialog box associated with the Graph Section

The resulting graph (Figure 8) shows the three plots (plate values with and without PathCheck and the cuvette values). The plot of raw plate absorbance values without PathCheck (bottom line) is irregular because the volumes in the wells (and therefore the pathlengths in the wells) were not uniform. The plot of plate values with PathCheck is linear through the highest concentration (6 mg/mL). The corrected plate values are superimposable on the cuvette values up through approximately 3 mg/ml, above which the cuvette plot begins to deviate

from linearity. The corrected plate plot extends above the cuvette plot because the values are calculated values and their raw absorbance values (bottom plot) are still well within the linear range of the curve.

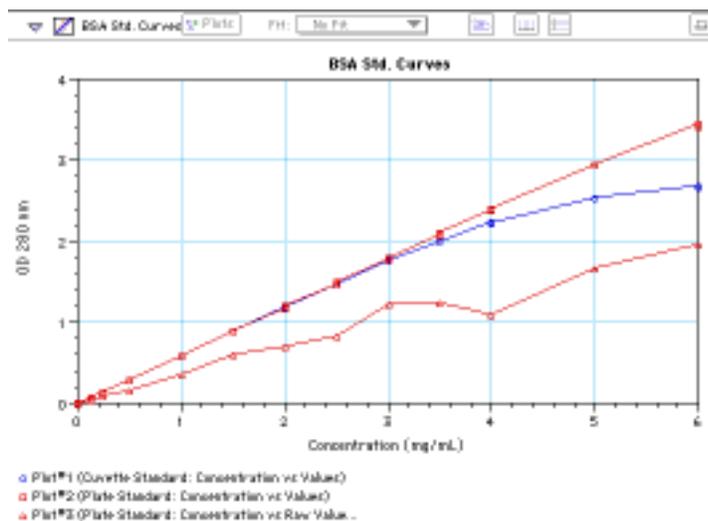


Figure 8: BSA standard curves obtained in a microplate and in a cuvette. The standards were read at 280 nm in a 1 cm pathlength cuvette (○) and in microplate wells with (□) and without (△) path-length correction

Example 2: Using PathCheck to evaluate column fractions during purification of antibody-enzyme conjugate on a gel filtration column

Fractions were collected during purification of an antibody-enzyme conjugate on a Pharmacia S-100 gel filtration column. The fractions in the void volume were discarded and fractions 21 through 61 were each read at 280 nm in a Costar UV plate and also in a standard quartz cuvette for comparison. Fractions 24 through 29 were too concentrated and therefore were diluted 1 to 5 to bring them into the linear range of absorbency.

Initial setup of the Group Table

There are two ways in SOFTmax PRO to set up a Group Table for samples (e.g. column fractions) that have dilution factors, but are not going to be quantitated against a standard curve:

- 1 Assign the 'Unknowns(dilution)' format to the group. SOFTmax PRO will automatically activate the Dilution Factor field and include columns for Dilution (Factor) and Adj.Result in the Group Table. The software also will assume a standard curve is being created, and will create 5 columns that are unnecessary for the present example (calculations of concentrations based on a standard curve and statistical information). Because these columns will not be needed in any calculations, you can delete them from the Group Table.
- 2 Assign the 'Basic' format to the group, then modify the group to include the dilution factor information, either via the Group Settings dialog box, or via the Series dialog box. Two columns must then be added to the Group Table: one that contains the dilution factors, and a second that multiplies the absorbance values by the dilution factors.

In this example, the second method was used to create the Group Table and assign sample names to the wells. A new group named "Plate" was created in the Plate section Template Editor and assigned the Basic format. Rows A through D were highlighted and the Series button clicked to access the Series dialog box (Figure 9).

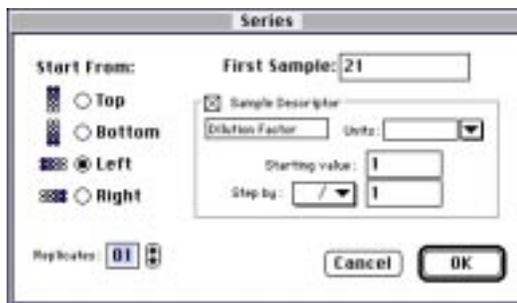


Figure 9: The Series Settings dialog box, set up for column fractions

The starting sample was set at 21 (to correspond to its fraction number) and the series was set to proceed from left to right with one replicate. The Sample Descriptor box was checked to activate it, then 'dilution factor' typed into the field. The Units field was cleared and left blank. After clicking the OK button to create the series, the template displayed the sample names (fraction numbers) assigned to the wells (Figure 10). Samples with dilution factors other than 1 had the dilution factor added by clicking the well in the template to select it, then typing in the appropriate factor into the Dilution Factor field. In the figure, well A5 (fraction #25) is highlighted and its sample name (25) and its dilution factor (5) are displayed in their respective fields. The buffer blanks were assigned to wells D6 and D7.

Note: the series feature initially filled all of Row D because SOFTmax PRO by default fills complete rows or columns. Wells D6 through D12 were cleared so that the series would stop at sample #61.

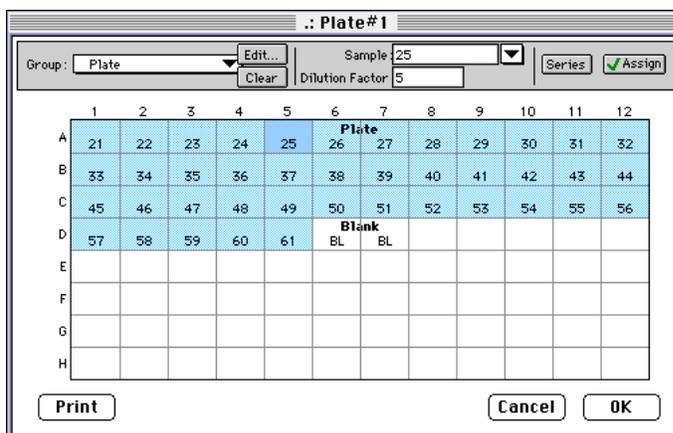


Figure 10: The Plate section Template Editor, showing the series set up

In an analogous manner, the same sample names were assigned to a CuvetteSet section.

Experimental details and customization of the group table

The instrument settings were identical to those used in Example 1 above. Plate pre-read measurements were made in a Costar UV plate. Aliquots of each column fraction and the buffer blank were transferred into their designated wells using disposable transfer pipets, then read in the SPECTRAmax PLUS. As in the example above, the volumes in each well were deliberately varied in order to demonstrate the utility of the PathCheck feature. The column fractions were also read in a quartz cuvette as described above.

The Group Table for the Plate section (after customizing) is shown in Figure 11. Of the seven columns, only three (Sample, Wells and Correct.Values) were created automatically by SOFTmax PRO. (The "Correct.Values" column was originally entitled "Values", but was renamed in this example to distinguish pathlength-corrected values from raw absorbance values. The other four columns were given custom formulas. The "Fraction#" column was created so that the samples could be given numeric values corresponding to their fraction numbers, then graphed. (In the Sample column, the sample numbers are treated as names and therefore cannot be graphed.)

Plate						
Sample	Well	Fraction#	Raw Values	Correct Values	Dilution	Final Value
21	A1	21	0.062	0.097	1.000	0.097
22	A2	22	0.063	0.092	1.000	0.092
23	A3	23	0.070	0.102	1.000	0.102
24	A4	24	0.067	0.099	5.000	0.496
25	A5	25	0.402	0.595	5.000	2.975
26	A6	26	0.818	0.960	5.000	4.800
27	A7	27	0.449	0.701	5.000	3.505
28	A8	28	0.369	0.514	5.000	2.569
29	A9	29	0.152	0.196	5.000	0.981
30	A10	30	0.061	0.096	1.000	0.096
31	A11	31	0.073	0.088	1.000	0.088
32	A12	32	0.177	0.249	1.000	0.249
33	B1	33	0.141	0.198	1.000	0.198
34	B2	34	0.062	0.098	1.000	0.098
35	B3	35	0.068	0.088	1.000	0.088
36	B4	36	0.057	0.098	1.000	0.098
37	B5	37	0.074	0.097	1.000	0.097
38	B6	38	0.127	0.205	1.000	0.205
39	B7	39	0.250	0.412	1.000	0.412
40	B8	40	0.302	0.451	1.000	0.451
41	B9	41	0.358	0.484	1.000	0.484
42	B10	42	0.270	0.369	1.000	0.369
43	B11	43	0.284	0.388	1.000	0.388
44	B12	44	0.311	0.365	1.000	0.365

Figure 11: The Group Table for the gel filtration column fractions read in the microplate

The custom formula for the Fraction # column (Figure 12) is "Index+20" so that the first entry is 21.

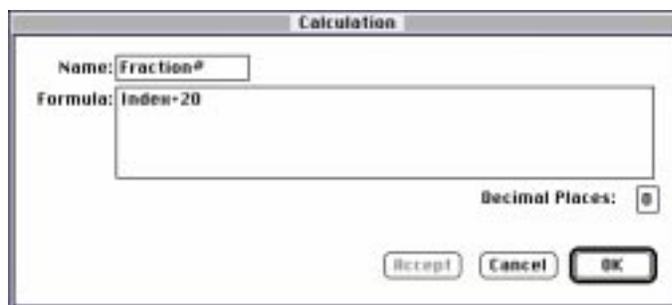
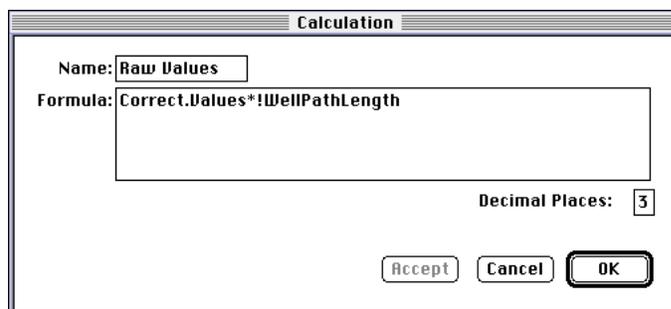


Figure 12: The Calculation dialog box for the "Fraction#" column

The "Raw Values" column contains the absorbance values before pathlength correction and were obtained by back-calculation from the corrected values using the

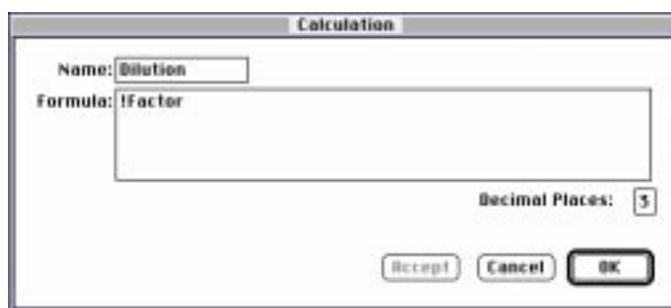
custom formula: "Correct.Values*!WellPathLength" (Figure 13).



The screenshot shows a dialog box titled "Calculation". It has a "Name:" label followed by a text box containing "Raw Values". Below that is a "Formula:" label followed by a larger text box containing "Correct.Values*!WellPathLength". To the right of the formula box is a "Decimal Places:" label followed by a small box containing the number "3". At the bottom of the dialog are three buttons: "Accept", "Cancel", and "OK".

Figure 13: The Calculation dialog box for the "Raw Values" column

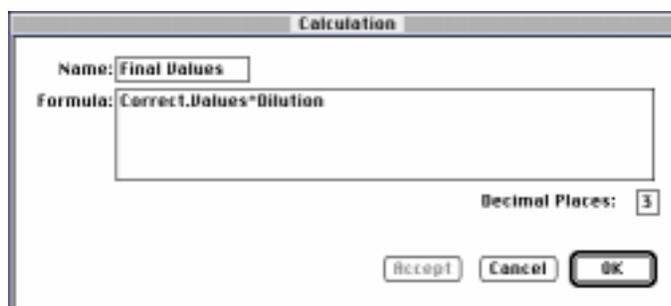
The custom formula for the "Dilution" column is shown in Figure 14. The term "!factor" refers to the sample descriptor defined in the template.



The screenshot shows a dialog box titled "Calculation". It has a "Name:" label followed by a text box containing "Dilution". Below that is a "Formula:" label followed by a larger text box containing "!factor". To the right of the formula box is a "Decimal Places:" label followed by a small box containing the number "3". At the bottom of the dialog are three buttons: "Accept", "Cancel", and "OK".

Figure 14: The Calculation dialog box for the "Dilution" column

The column "Final Values" contains the corrected absorbance values multiplied by the dilution factor. The custom formula is Correct.Values*Dilution (Figure 15). In the Cuvette Group Table, an analogous column was created to multiply the absorbance values by the dilution factors.



The screenshot shows a dialog box titled "Calculation". It has a "Name:" label followed by a text box containing "Final Values". Below that is a "Formula:" label followed by a larger text box containing "Correct.Values*Dilution". To the right of the formula box is a "Decimal Places:" label followed by a small box containing the number "3". At the bottom of the dialog are three buttons: "Accept", "Cancel", and "OK".

Figure 15: The Calculation dialog box for the "Final Values#" column

Figure 16 shows the elution profiles from the gel column obtained in the plate and the cuvette. The lower plot (plate values not pathlength corrected), not surprisingly, does not strictly mirror the upper profiles because the volumes in the wells were not uniform. However, when PathCheck was applied to the plate values, the

plate profile was essentially superimposable on the cuvette profile.

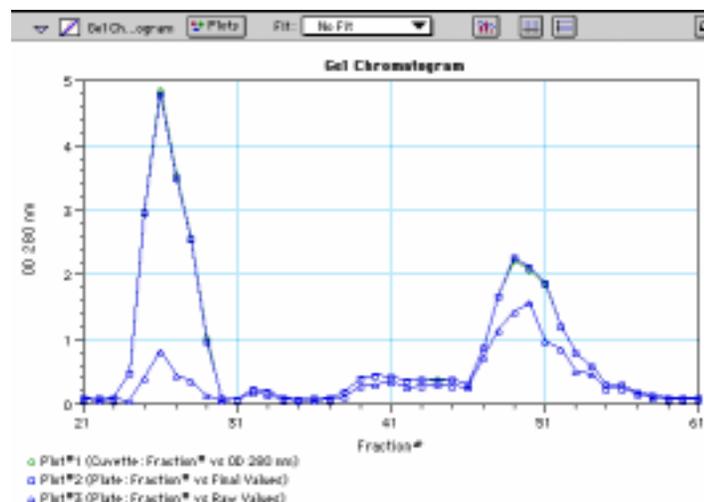


Figure 16: Elution profile of column fractions obtained during purification of antibody-enzyme complex. The fractions were read at 280nm in a 1 cm pathlength cuvette (○), and in microplate wells with (□) and without (△) pathlength correction

The above examples illustrate the utility of the PathCheck feature of the SPECTRAmax PLUS. Protein solutions can be read in a microplate and the absorbance values are identical to those obtained by reading the same solutions in a standard 1 cm cuvette. Accurate pipetting is not necessary. An additional benefit of PathCheck is that the linear range is extended above the cuvette linear range (as long as the raw absorbance values in the wells are within the linear range of the instrument).

SALES OFFICE

United States & Canada
Molecular Devices
Tel. +1-800-635-5577
Fax +1-408-747-3601

Brazil
Molecular Devices Brazil
Tel. +55-11-3616-6607
Fax +55-11-3616-6607

China
Molecular Devices Shanghai
Tel. +86-21-6887-8820
Fax +86-21-6887-8890

Germany
Molecular Devices GmbH
Tel. +49-89/96-05-88-0
Fax +49-89/9-62-02-34-5

Japan
Molecular Devices Japan, Osaka
Tel. +81-6-6399-8211
Fax +81-6-6399-8212

Molecular Devices Japan, Tokyo
Tel. +81-3-5282-5261
Fax +81-3-5282-5262

South Korea
Molecular Devices Korea, LLC
Tel. +82-2-3471-9531
Fax +82-2-3471-9532

United Kingdom
Molecular Devices Ltd.
Tel. +44-118-944-8000
Fax +44-118-944-8001

www.moleculardevices.com

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