Userguide

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Generation of a standard curve for a colorimetric assay in the Eppendorf BioSpectrometer® basic and Eppendorf BioSpectrometer® kinetic

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Abstract

In order to demonstrate the development of a measurement method for a colorimetric assay in the Eppendorf BioSpectrometer, Ponceau-S was scanned across a range of wavelengths from 220 nm to 800 nm to determine its absorbance maxima.
Using the novel analysis methods available on the Eppendorf BioSpectrometer, a maximum could be detected at 498 nm using the Eppendorf "SpectraZoom" function.
For the colorimetric assay, a standard curve was generated using Ponceau-S as an example, and measurements were performed. Subsequently, this programmed standard curve was used to calculate an unknown sample concentration.

Introduction

Example methods for colorimetric assays

Colorimetric assays allow for indirect determination of specific substrate concentrations, such as proteins or carbohydrates, as well as determination of enzyme activity, via a color change. These reactions can be performed directly inside the spectrophotometer.
In principle, all measurements occur in the visible range of light (approx. 380 nm - 780 nm).
Examples include colorimetric protein determination (Bradford, Lowry, BCA), cytotoxicity assays, detection of fructose in seminal plasma [1] as well as enzymatic determinations, such as phosphatase assays, alpha glucosidase assays or beta-galactosidase assays [2], to name a few.

Colorimetric assays for protein determination

With the Bradford method of protein determination, protein is first stained using Coomassie brilliant blue G-250. During this process, a dye-protein complex is formed in an acidic environment. During the formation of this complex, the solution experiences a color change to blue; the intensity of the color is proportional to the concentration of protein in solution. Thus, protein concentration may be indirectly measured by determining color intensity by way of photometry (fig. 1).

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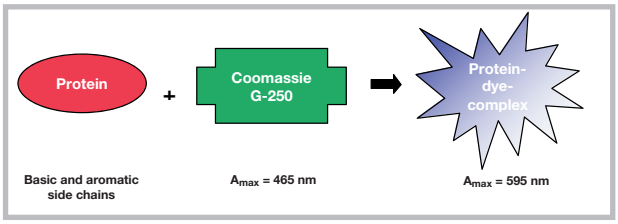

Figure 1: Principle of protein staining following the Bradford method.
The formation of the protein-dye complex changes the absorbance maximum of the dye from 465 nm to 595 nm. Following a short incubation period of 5-10 min, the protein-dye complex remains stable and it may be measured in the spectrophotometer at 595 nm.

Colorimetric assays for the determination of enzyme activity

For this purpose, kinetic measurements via the end point method are discussed, where substrate conversion by the enzyme is stopped after a clearly defined period of time has elapsed. An artificial substrate is used for the enzymatic reaction, during which a dye molecule (chromophore) is released following cleavage by the enzyme. This process can be detected photometrically. The activity of the enzyme can thus be determined via the amount of liberated dye and the incubation time of the enzyme reaction. Figure 2 shows the example of the reaction of an α-glucosidase with para-nitrophenyl-α-D-glucopyranoside.

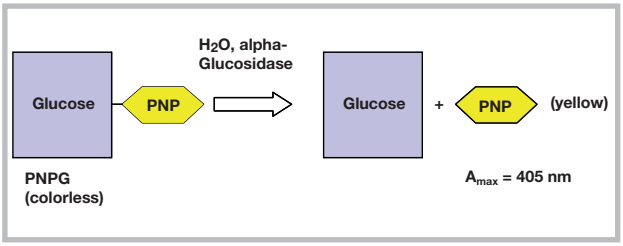

Figure 2: Reaction of an α-glucosidase with para-nitrophenyl-α-D-glucopyranoside. The liberation of para-nitrophenol (PNP) is detectable via a color change of the solution to yellow. The reaction is stopped by the addition of an alkaline sodium carbonate solution.

Evaluation of colorimetric tests in the spectrophotometer

All colorimetric detection methods have in common the principle that an unknown sample concentration or the enzymatic activity of an unknown sample, is determined using a calibration curve. In order to create a calibration curve, different known standards are measured in succession using a spectrophotometer. Subsequently, regression analysis is performed; i.e. the available values obtained are used to define an appropriate regression curve which most closely overlaps with these values.

The calibration curves generated in this fashion can follow either a linear (fig. 3) or a non-linear (fig. 4) course.

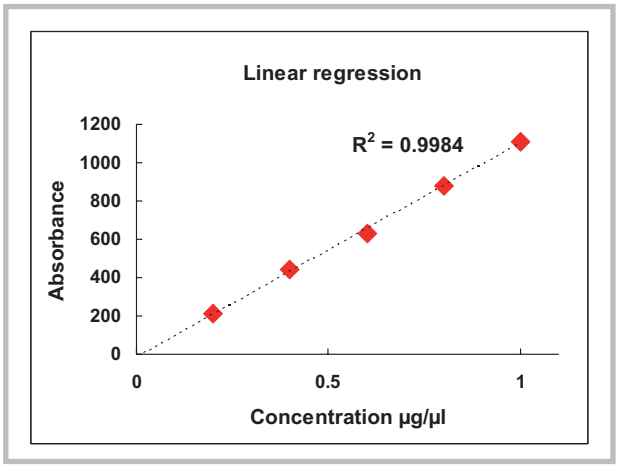

Figure 3: Standard curve with linear shape.
Example: fructose-determination [1]

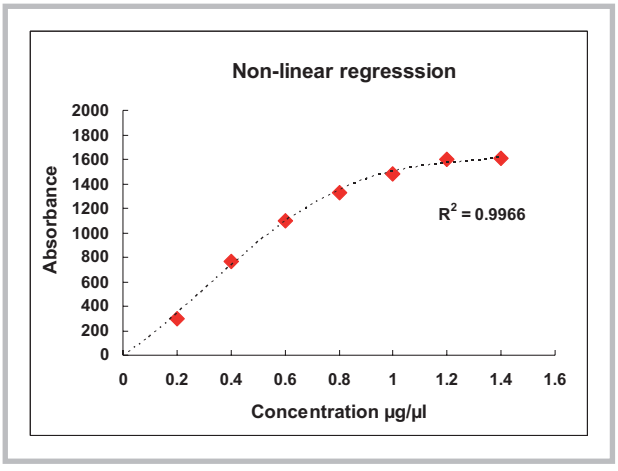

Figure 4: Standard curve with non-linear regression.
Possible shape of a Bradford standard curve

One important factor determining the accuracy of the regression curve is the coefficient of determination R^2 (as shown in figures 3 and 4). The closer the R^2 value is to 1, the closer the regression curve overlaps with the actual values measured. For measurements performed in the Eppendorf BioSpectrometer, this value should not be smaller than 0.95.
Fortunately, the Eppendorf BioSpectrometer allows for direct storage of measured values in the instrument, which may be recalled at a later time for subsequent measurements. Regression analysis occurs automatically using the values obtained.

Furthermore, the coefficient of determination R^2 is always calculated and displayed automatically.
The Eppendorf BioSpectrometer offers 3 regression analysis techniques and 2 interpolation techniques:

- linear interpolation
- linear regression
- quadratical regression
- cubical regression
- spline interpolation

The interpolation techniques are available for curve shapes which are not analyzed using regression techniques. During spline interpolation, the individual standard values are connected by overlapping sections of curve functions; this process is especially suited for S-shaped curves. During linear interpolation, the individual standard values are connected by sections of linear functions; this process is the method of choice if none of the other options yield satisfactory results.
Usually, the shape of the expected curve is known in advance, and the appropriate analysis technique may be selected. If this is not the case, the best fitting analysis method needs to be researched.
The present article shows the generation of a standard curve on the Eppendorf BioSpectrometer, using the dye Ponceau-S, and subsequent analysis of the saved values via regression analysis, for the purpose of determination of unknown sample concentrations. First, a wavelength scan was performed with a pre-determined Ponceau-S-concentration, in order to determine the exact absorbance maximum of this dye.
For this purpose, the user-friendly analysis functions of the Eppendorf BioSpectrometer were used, and a peak at 331 nm and 498 nm, respectively, could be determined. For the standard curve, measurements were subsequently performed at 498 nm.

Materials

Instruments: Eppendorf BioSpectrometer basic or kinetic
Materials:
0.01 % (w/v) Ponceau-S solution
Water
Eppendorf UVettes

Execution and results

Determination of maximum absorbance of the Ponceau-S dye
For the purpose of determination of the absorbance maximum of the Ponceau-S dye, a preliminary wavelength scanis to be performed between 220 and 800 nm, using the BioSpectrometer. To this end, “Absorbance” is selected from the main menu, followed by the subgroup “Scan”. Here, the method “Scan” of the same name is selected (fig. 5).

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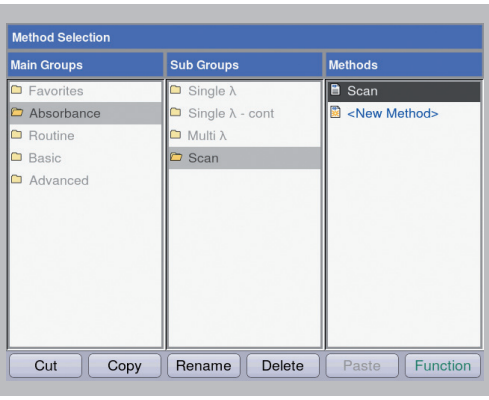

Figure 5: Selection of the method “Scan” for spectroscopic analysis of the dye Ponceau-S using the Eppendorf BioSpectrometer.

Subsequently, the scan parameters are determined. The soft key “Edit” in the area “Check parameters” allows for adjustment of the wavelength-range and the path length of the cuvette (fig.6).

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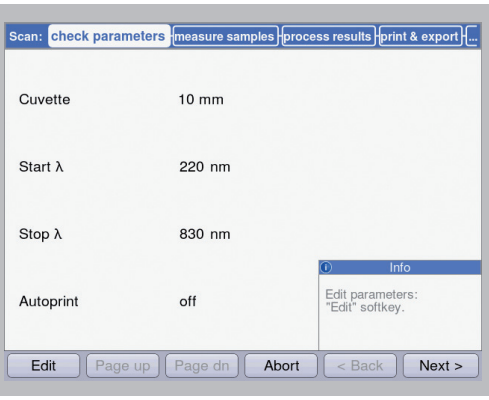

Figure 6: Measurement parameters for the wavelength scan of the dye Ponceau-S.

After all parameters have been selected, the soft key “Next” leads to the area “Measure samples”. For the wavelength scan, water is first measured as a blank value, followed by a 0.01% (w/v) Ponceau-S solution as the sample (fig.7).

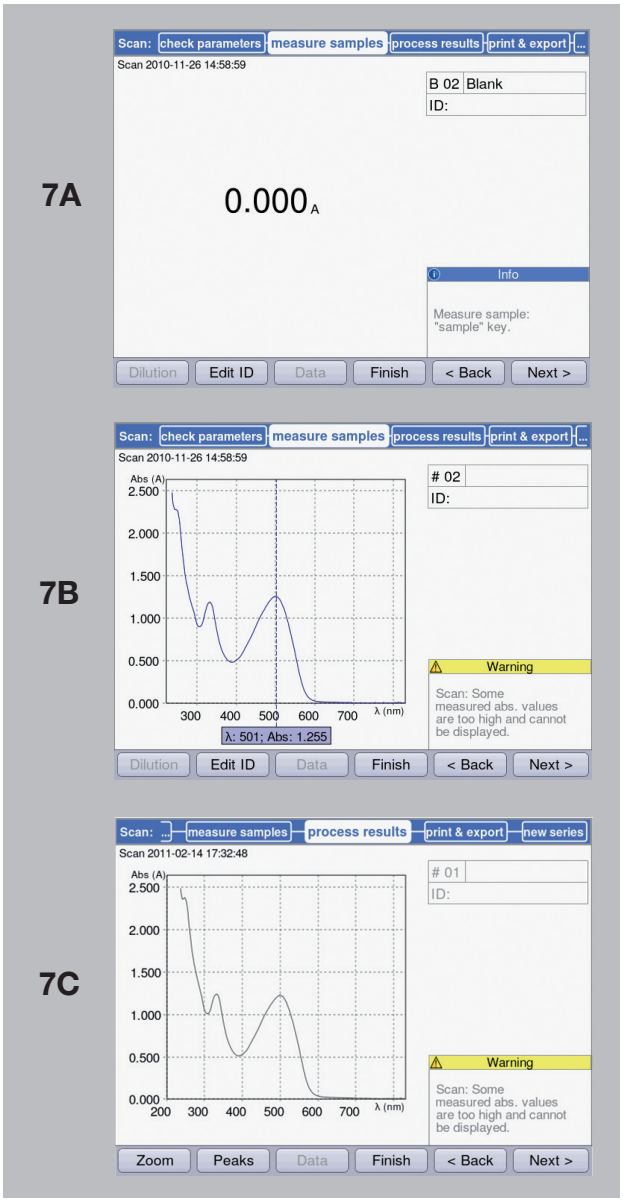

Figure **7A** Blank measurements for the scan.
7B Wavelength scan of a 0.01% (w/v) Ponceau-S solution between 220 and 800 nm.
7C Process results: Peak detection ("Peaks") or zoom function ("Zoom").

As shown in figure 7B, two peaks are obvious in this spectrum. Activation of the "Next" soft key leads to the area "Process results". Here, the option of identifying the exact wavelengths of the absorbance maxima is available (Fig 7C). The Eppendorf BioSpectrometer offers two functions which fulfill this purpose: the function "SpectraZoom" (Soft key "Zoom") and the function "Peak detection" (Soft key "Peaks").

Spectrum analysis via the "Zoom" function

The zoom in the area "Process results" allows for analysis of the collected spectrum using 3 different functions: "spectra", "spectra-0", "free" (fig.8).

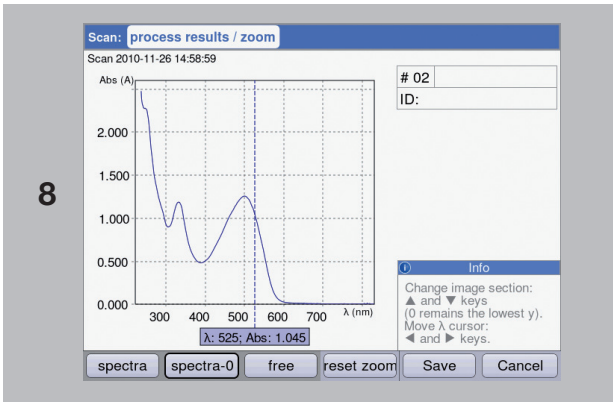

Figure 8: Possible manipulation of the spectrum with "Zoom" function:
1) Function "spectra": Magnification of the section using the cursor buttons in the area of the wavelength indicator (dashed line).
2) Function "spectra-0": Same as function "spectra", except that Abs(y)=0.
3) Function "free": Free entry of a wavelength (x-axis) and absorbance range (y-axis)

As shown in figure 8, the selection of the spectrum containing the wavelength indicator may be enlarged using the cursor buttons "up" and "down" (Eppendorf SpectraZoom). If the function "spectra-0" is selected, the zero value is always displayed on the Y-axis (absorbance). Using this method, the absorbance maximum at 498 nm for the dye Ponceau-S could be determined quickly and simply (fig.9).

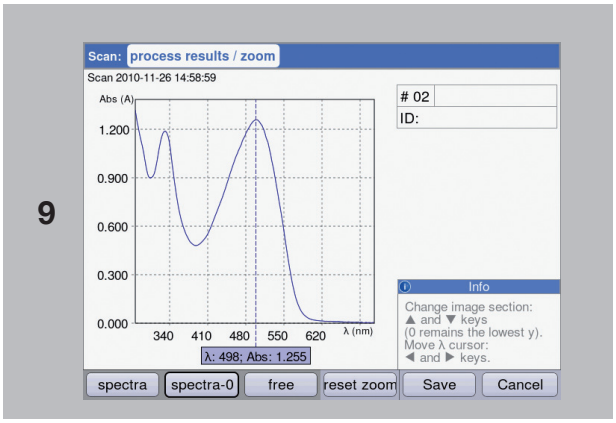

Figure 9: Identification of the absorbance maximum of Ponceau-S at 498 nm using the SpectraZoom function.

As shown in figure 9, an additional peak was clearly visible at 330 nm. Exact identification of the peaks within the spectrum is to be demonstrated below, using the function "Peak detection".

Spectrum analysis via “Peak detection”
The Eppendorf BioSpectrometer offers a further method for spectrum analysis: peak detection. This function is activated by the soft key “Peaks” within the area “Process results”.

In order to identify absorbance maxima within a spectrum using this function, the wavelength grid and the absorbance range contained therein, may be enlarged or shrunk, by activating the cursor buttons “left”, “right”, or “up”, “down”, respectively. To illustrate this function, the details of the Ponceau-S spectrum are shown in figures 10 A-C.
Preliminary settings for the function “Peak detection” are displayed in figure 10A. The wavelength grid “λ-grid” is set to 10 nm. The difference between lowest and highest absorbance may not be lower than 0.4 (Min. Δ abs = 0.4).
This means that within an area of +/- 5 nm, a difference in absorbance of at least 0.4 needs to be present in order to identify a peak within a spectrum. The following figures 10B and 10C demonstrate what happens when the grid is expanded step-wise around the Ponceau-S spectrum.

The value of the difference in absorbance is not changed. Figure 10B shows that this way, a peak is identified at 331 nm. With repeated enlargement of the grid to 78 nm, a further peak is identified at 498 nm (fig. 10C). By activating the soft key “Peak table”, all peaks thus identified are displayed accurately, showing their exact absorbance (fig. 10D).

As shown in figure 10, the largest peak at 498 nm was detected using “Peak detection”. Therefore, the subsequent measurements for the standard curve were performed at this wavelength.

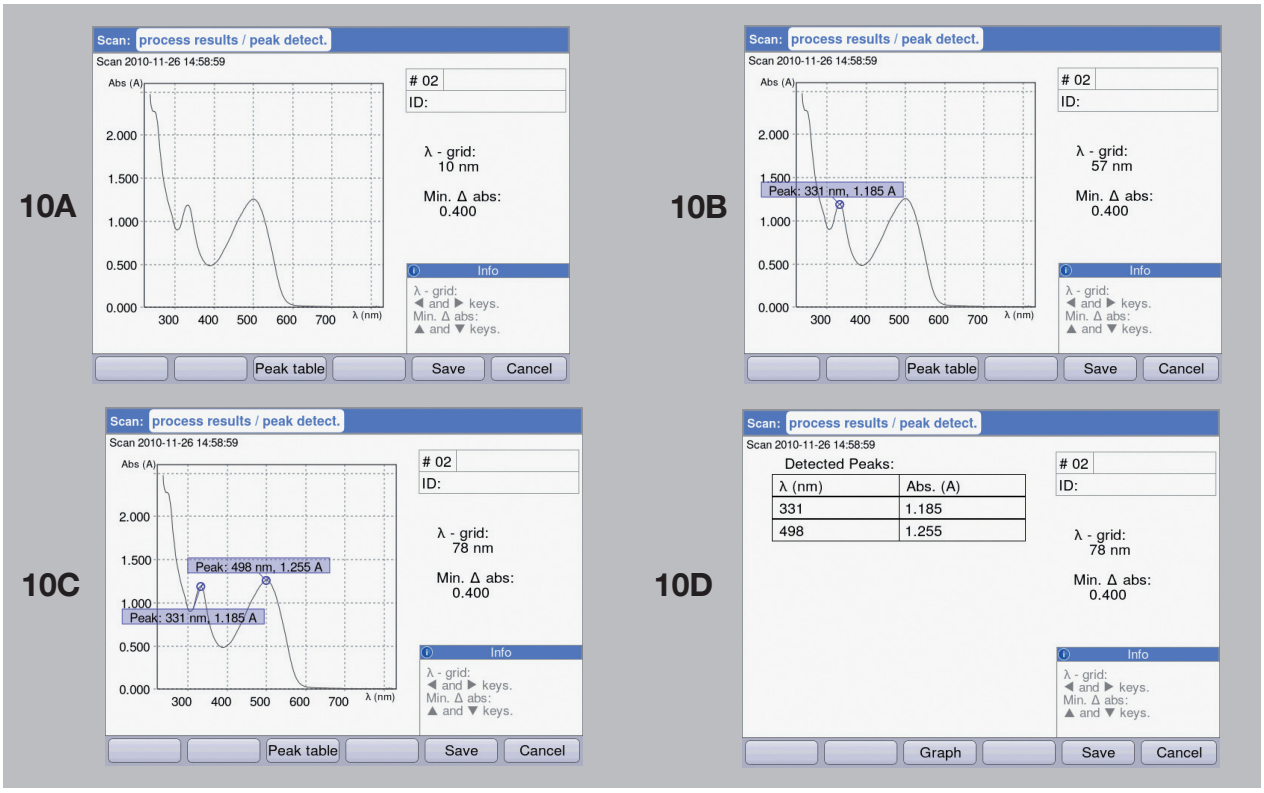

Figure 10: Spectrum analysis using “Peak-Detection”:
The wavelength grid (“λ-grid”) is altered using the cursor buttons “right” and “left” while the difference in absorbance remains unchanged (Min. Δ abs = 0.4):

10A No peak is detected within the spectrum inside a grid of 10 nm, i.e. in no area within the spectrum are differences in absorbance of 0.4 detected within a range on 10 nm.
10B A peak at 331 nm is identified when a grid of 57 nm is applied.
10C When the grid is expanded to 78 nm, a further peak is identified at 498 nm.
10D Overview table showing all peaks detected.

Generation of a standard curve for the purpose of photometric analysis of a colorimetric assay

The following section shows how different standards are measured in the BioSpectrometer for the purpose of generating a standard curve. To this end, 10 standards with defined concentrations of a Ponceau-S solution are prepared, where the Ponceau-S solution simulates the highest dye concentration (1000 mg/mL).

Table 1 represents an overview of all standards to be measured.

Table 1: Concentrations of standard solutions

Number	Concentration [mg/mL]
1	100
2	200
3	300
4	400
5	500
6	600
7	700
8	800
9	900
10	1000

For the measurement of standards in the Eppendorf BioSpectrometer, the method “Calibration curve” is activated (fig. 11).

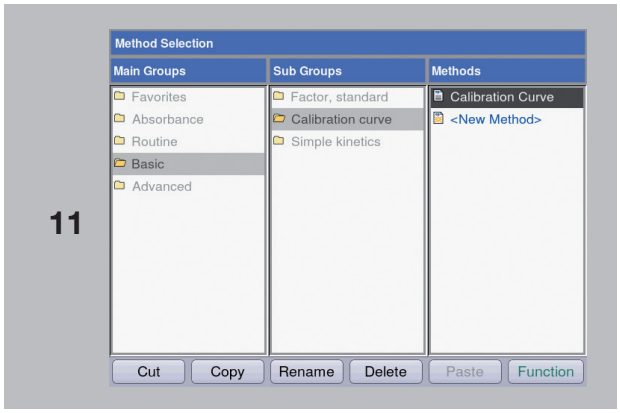

Figure 11: Selection of the method “Calibration curve”

Within this method, all parameters required for the generation of a standard curve are defined. Parameters may be changed using the soft key “Edit” (fig. 12)

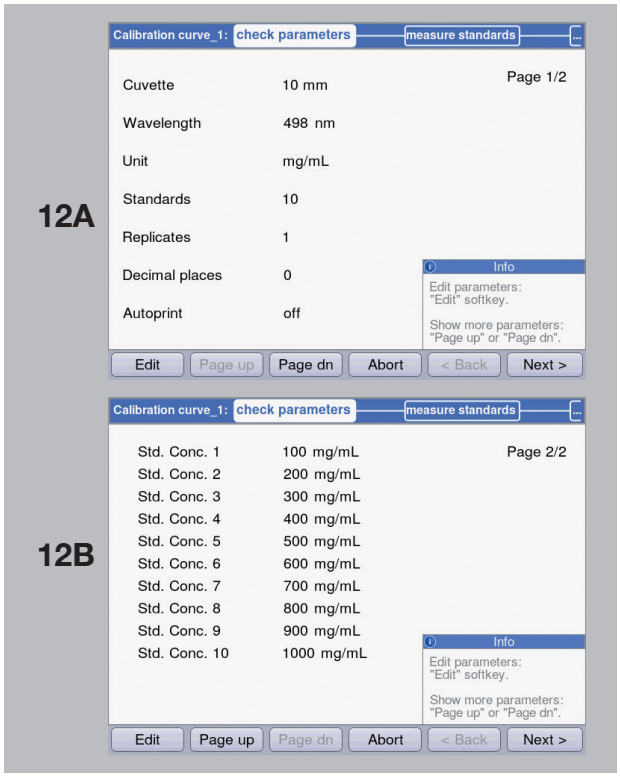

Figure 12: Selection of the method “Calibration curve”

Following programming of all standards, the soft key “Next” leads to the area “Measure standards”. Here, all standards may be measured in succession or in any order of choice. It is further possible to repeat measurements if needed. Measurement of a standard is always carried out using the button “Standards”.

The soft key “Curve fit” defines the nature of standard analysis for the values obtained. This function may be selected and changed at any time during the measurement process. As long as a sufficient number of values is available, calculations for curve analysis are automatically performed, and, in the case of regression analysis, the coefficient of determination is automatically displayed.

Figure 13 shows the change of the coefficient of determination following the alteration of the nature of the regression. When a sufficient number of standards are available for an analysis, the area of standard evaluation may be left, and the determination of unknown sample concentrations may be commenced.

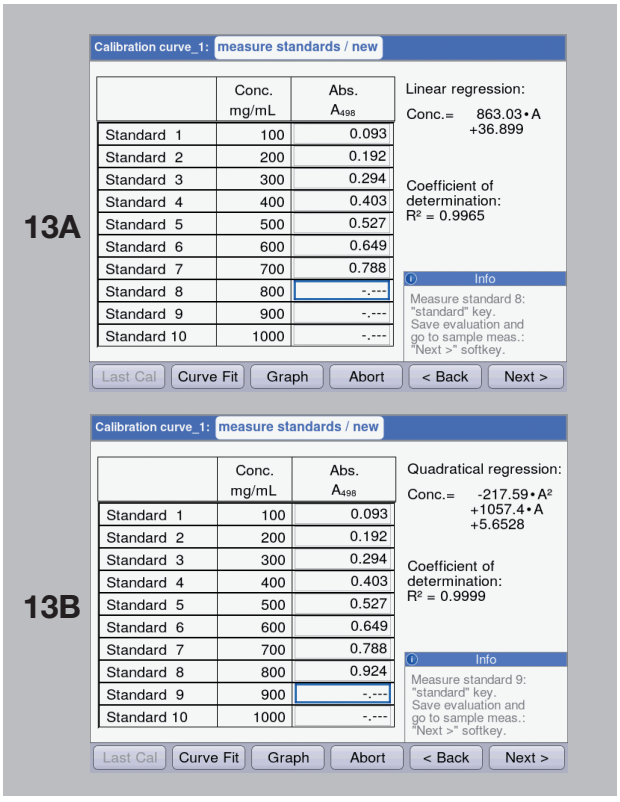

Figure 13: Programming of the parameters required for the measurement of a standard curve (A) and definition of the standard concentrations to be measured (B).

As shown in figure 13, the coefficient of determination could be improved slightly by a switch from linear regression to quadratical regression. This is reflected in the resulting curves following the measurement of all standards (fig. 14).

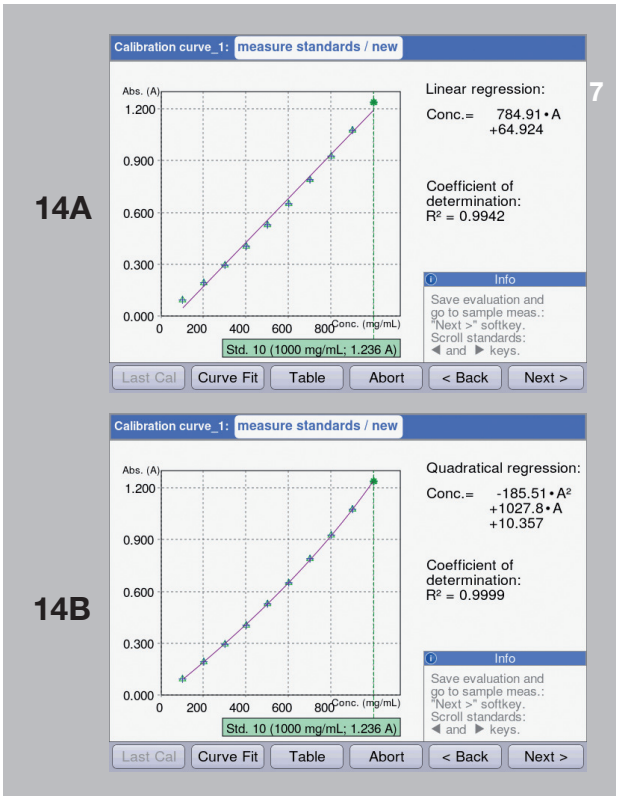

Figure 14: Change of regression analysis from linear (A) to quadratical (B).

Once the standard curve has been established, sample measurement may be initiated immediately. The soft key “Next” leads directly into the measurement area (“Measure samples”).
An example of sample measurement is shown in figure 15.

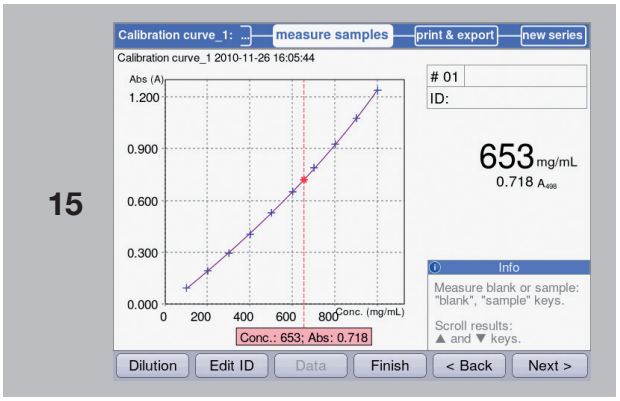

Figure 15: Sample determination via standard curve.
The result is directly linked to the generated standard curve

As shown in figure 15, the result of sample measurement is shown directly within the standard curve. This feature demonstrates that the range of the standards was selected correctly for the determination of this sample.

Conclusion

The Eppendorf BioSpectrometer allows quick and simple determination of absorbance maxima within a spectrum using zoom function and peak detection. This way, the exact wavelength at which a dye is to be measured in a colorimetric assay is easily determined.
Programming and measurement of standards are quick and simple; individual standards may be measured repeatedly as often as desired, and curve analysis techniques may be fitted retroactively prior to saving of the analysis method, initiated by the switch to sample measurement. 5 different analysis techniques are available.
The sample measurement result is displayed directly within the standard curve in order to facilitate evaluation of the correctness of the standard curve range.

Literature

[1] *Eppendorf UserGuide No.33*: Colorimetric determination of fructose using standard curves at 490 nm in the Eppendorf BioPhotometer plus. www.eppendorf.com

[2] Christin Buro, Svenja Beckmann, Thomas Quack and Christoph G. Grevelding. *Eppendorf Application Note 221*: Photometric quantification of the β-Galactosidase activity for the analysis of the relative interaction strengths between signal transduction proteins from *Schistosoma mansoni* in the Eppendorf BioPhotometer plus. www.eppendorf.com

Ordering information

Product	Description	Order no. International	Order no. North America
Eppendorf BioSpectrometer® basic	230 V / 50 - 60 Hz, mains/power plug Europe	6135 000.009	-
	120 V / 50-60 Hz, mains/power plug North America	-	6135000017
Eppendorf BioSpectrometer® kinetic	230 V / 50 - 60 Hz, mains/power plug Europe	6136 000.002	-
	120 V / 50-60 Hz, mains/power plug North America	-	6136000010
Thermo printer DPU 414	Incl. power supply and printer cable 230 V	6131 011.006	-
	Incl. power supply and printer cable 120 V	-	952010140
Thermo paper	5 rolls	0013 021.566	952010409
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