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Factor based quantification of nucleic acid concentrations in the Eppendorf PlateReader AF2200 using the Eppendorf Microplate UV-VIS

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Abstract

In photometers, concentrations of nucleic acids, for example, are easily determined with the help of Lambert-Beer's law, taking into consideration the path length of the cuvette, the sample-specific extinction factor and the absorbance value (at 260 nm for nucleic acids). This is not the case for determination of sample concentrations in microplates; it is generally required to generate a standard curve which is then used to determine the concentration of an unknown sample. This Application Note will demonstrate how concentration determination via the sample-specific extinction factor is possible with the Eppendorf PlateReader AF2200 in combination with the Eppendorf Microplate UV-VIS. This approach eliminates the need for a standard curve, thus saving both time and money. The results obtained are compared to reference measurements in a standard cuvette as well as to the established method of using a standard curve.

Introduction

Determination of concentration via a sample-specific extinction factor is a common approach for the cuvette format, but not for the plate format. The underlying calculation is Lambert-Beer's law [1].

$$A = \varepsilon * c * d$$

For this calculation, knowledge of the sample specific factor, as well as the exact path length are required. In the case of cuvettes, the path length is determined by the shape of the cuvette, as the light beam traverses the cuvette horizontally. In the case of absorbance measurements in a plate, the light beam traverses the sample vertically.

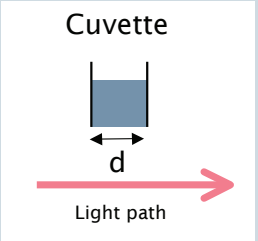

Figure 1: Light beam through a cuvette. The light path length is defined by the shape of the cuvette (diameter).

If a microvolume plate such as the Eppendorf µPlate G0.5 is used, the distance between the upper and the lower side of the plate (or the quartz wells, respectively) will determine the path length. In the case of the Eppendorf µPlate G0.5 this distance, and therefore the path length, is 0.5 mm (G = gap 0.5 mm). This feature considerably simplifies concentration determination via extinction factor on the microvolume scale.

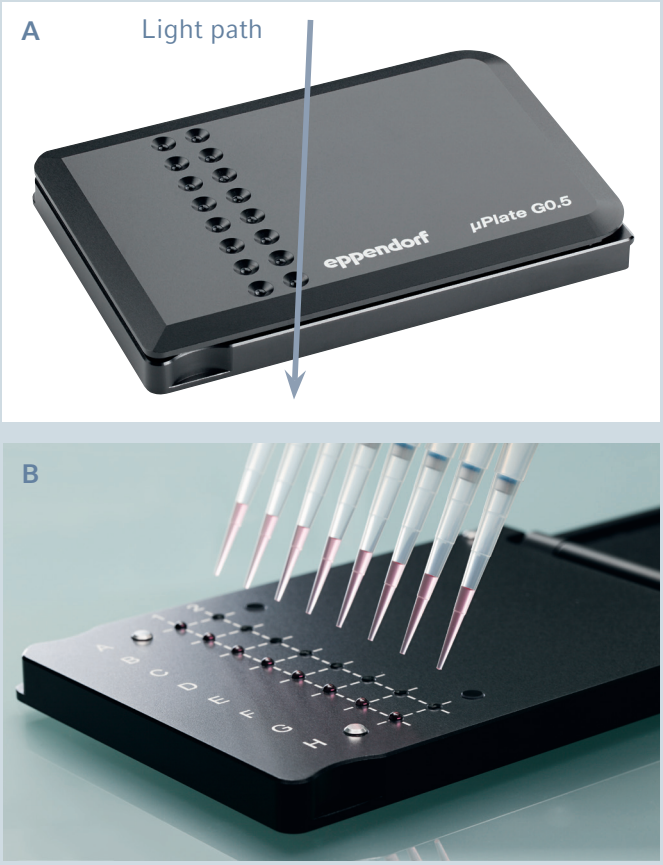

Figure 2: **A:** Light beam through the Eppendorf µPlate G0.5.
B: Pipetting of 8 samples on the Eppendorf µPlate G0.5 using an 8-channel pipette.

The situation is more complex when microplates with 96 or 384 wells are concerned. In this case, the filling height determines the path length. Since the filling height is not only dependent on the volume but also on the geometry of the well, exact determination of the filling height is critical.

Use of the Eppendorf Microplate UV-VIS with defined volume enables this type of concentration determination in the PlateReader AF2200. Since the exact dimensions of the plates and wells are known, the software already contains the respective path lengths for pre-defined filling volumes. Calculation of sample concentration is performed analogous to the familiar process employed for instruments using cuvettes. Exact pipetting is of course a prerequisite to ensure uniform filling heights. Of equal importance is the use of high quality microplates with the lowest possible tolerance values between wells and also between plates.
This precondition is of course fulfilled by the Eppendorf Microplate UV-VIS.
In addition, the sample itself influences the reproducibility of results. For example, detergent-containing buffers may form a strong meniscus at the surface, which will influence filling height.

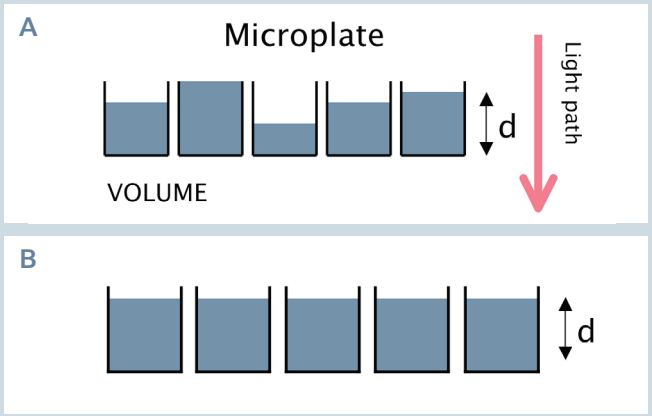

Figure 3:
A: Different liquid levels due to unreproducible pipetting or high variances in the well geometry.
B: Equal liquid levels and thus reproducible light path lengths (d).

Materials and Methods

Materials
> Novagen® herring sperm DNA (Merck® Millipore®)
> Quartz cuvettes with 10mm path length (Hellma® Analytics)
> Cary® 100 UV-Vis Spectrophotometer (Agilent®, Agilent Technology)
> Eppendorf Microplate UV-VIS with UV-transparent film bottom (eppendorf®)
> Eppendorf PlateReader AF2200 (eppendorf®)
> Tris buffer 0.1 M, pH 8.0

Methods
The herring sperm DNA is diluted in Tris buffer in order to generate solutions containing the following concentrations:
> 100 ng/ µL (stock solution)
> 80 ng/ µL
> 60 ng/µL
> 50 ng/µL
> 25 ng/µL
> 10 ng/µL
> 5 ng/µL

The concentrations of the different dilutions are determined in a quartz cuvette using the Cary 100, at a wavelength of 260 nm. The Tris buffer serves as the blank. Absorbance at 260 nm is measured for all dilutions from 5 replicates, and the averages and standard deviations are calculated.

Table 1: Raw data and calculated concentrations of the different DNA dilutions, measured in a quartz cuvette (10mm path length) on the Cary 100.

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	2.082	0.00023	104.1
80	1.654	0.00008	82.7
60	1.238	0.00011	61.9
50	1.033	0.00016	51.7
25	0.512	0.00019	25.6
10	0.203	0.00009	10.1
5	0.100	0.00016	5.0

Subsequently, the different concentrations are pipetted into the Micoplate UV-VIS. Care is taken to pipet in a highly reproducible fashion and to avoid splashes on the well rim, for example. Three plates are filled with different volumes (100 µL / 200 µL / 300 µL) of each dilution. Again, the Tris buffer serves as the blank. Each plate is first measured using the method "Nucleic Acid quantification (UV 260nm with standards)", with the following concentrations measured as standards:
> 100 ng/ µL
> 50 ng/µL
> 25 ng/µL
> 5 ng/µL

The concentrations 80 ng/µl, 60 ng/µL and 10 ng/µL are then determined via the standard curve. Each dilution and each blank measurement are determined in triplicate. Subsequently, all concentrations are again determined using the factor-based method "Nucleic Acid quantification (UV 260 nm with factor)" and compared to the results obtained with the standard curve, as well as with those obtained from the cuvette measurements on the Cary 100.
During the course of factor-based measurements, absorbance values at the following wavelengths, besides 260 nm, were determined: absorbance at 280 nm and 340 nm. The value generated at 340 nm is taken into consideration as a reference value in order to minimize the influence of contamination. The absorbance measurement at 260 nm is used to calculate the DNA concentration following Lambert-Beer's law. Calculation of concentration is performed by the instrument's software by using the sample specific extinction factor of 50 for double-stranded DNA (dsDNA). This factor states that an absorbance value of 1, measured in a 10 mm light path, translates to a DNA concentration of 50 µg/µL [1].

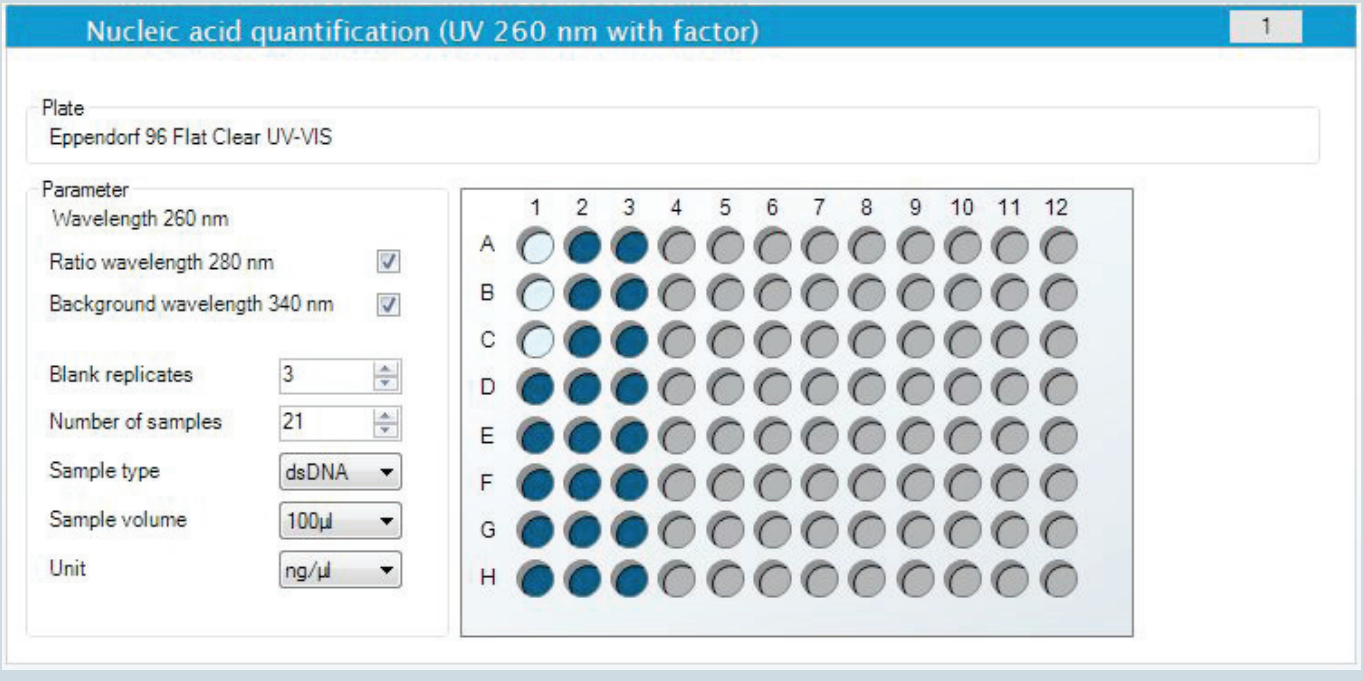

Figure 4: Screenshot of the method "Nucleic Acid quantification (UV 260nm with factor)" from the software of the PlateReader AF2200.

Results

Table 2: 100 µl sample: analysis via standard curve

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	0.7342	0.00501	Standard
80	0.1346	0.00182	78.96
60	0.4635	0.00040	59.50
50	0.4000	0.00602	Standard
25	0.2324	0.00135	Standard
10	0.5935	0.00461	10.25
5	0.1001	0.00032	Standard

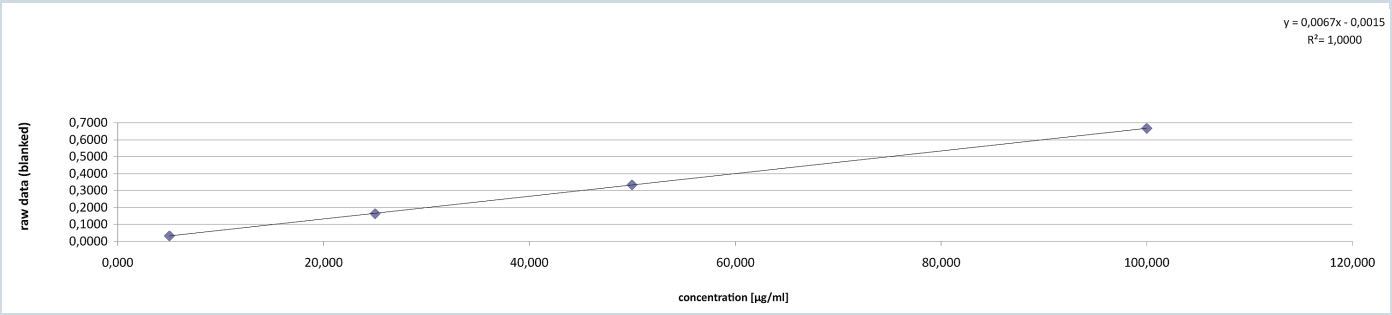

Figure 2: 100 µl sample: standard curve

Table 3: 200 µl sample: analysis via standard curve

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	1.4300	0.00718	Standard
80	1.1456	0.00130	79.30
60	0.8780	0.00380	59.73
50	0.7425	0.00341	Standard
25	0.4019	0.00469	Standard
10	0.1969	0.00191	9.91
5	0.1322	0.00025	Standard

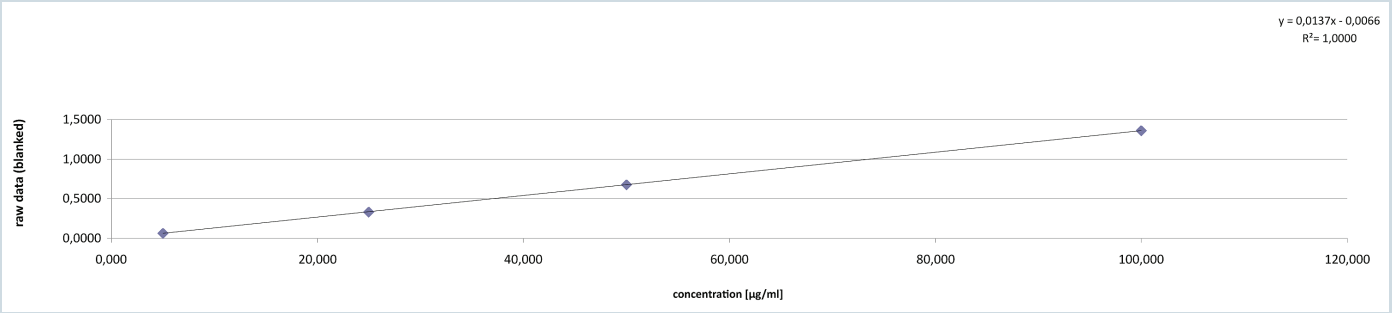

Figure 3: 200 µl sample: standard curve

Table 4: 300 µl sample: analysis via standard curve

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	2.0841	0.01360	Standard
80	1.6626	0.00733	79.21
60	1.2671	0.00889	59.63
50	1.0705	0.00907	Standard
25	0.5645	0.00414	Standard
10	0.2702	0.00542	10.25
5	0.1680	0.00231	Standard

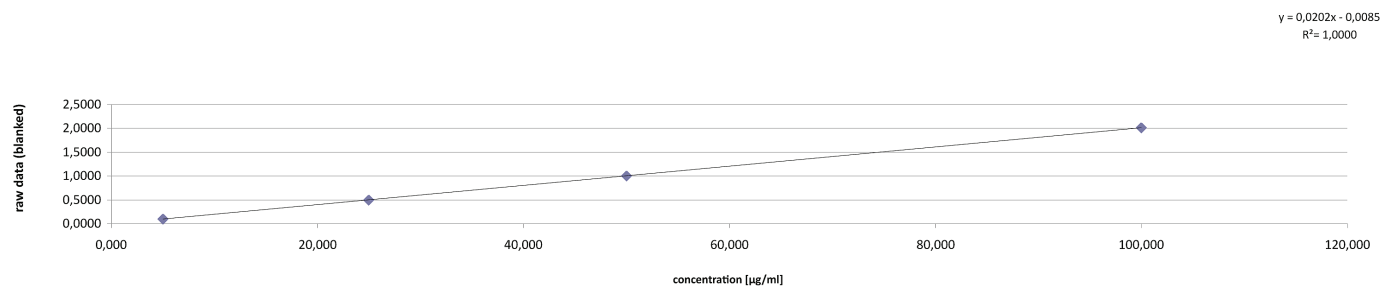

Figure 4: 300 µl sample: standard curve

Table 5: 100 µl sample: analysis via sample-specific extinction factor

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	0.7465	0.00365	99.81
80	0.6061	0.00726	79.31
60	0.4697	0.00199	59.18
50	0.4048	0.00525	49.71
25	0.2332	0.00089	24.38
10	0.1336	0.00146	9.75
5	0.0999	0.00079	4.91

Table 6: 200 µl sample: analysis via sample-specific extinction factor

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	1.4343	0.00091	101.89
80	1.1500	0.00747	80.72
60	0.8764	0.00785	60.12
50	0.7420	0.00341	50.25
25	0.4018	0.00344	24.76
10	0.1972	0.00159	9.58
5	0.1330	0.00046	4.73

Table 7: 300 µl sample: analysis via sample-specific extinction factor

Prepared DNA concentration in ng/µL	Average absorbance at 260nm	Standard deviation of absorbance at 260nm	Calculated concentration in ng/µL
100	2.1036	0.01086	102.60
80	1.6768	0.01148	81.10
60	1.2748	0.00878	60.75
50	1.0727	0.00881	50.55
25	0.5667	0.00462	24.94
10	0.2654	0.00206	9.72
5	0.1674	0.00055	4.82

Table 8: 100 µl sample: Concentrations determined via cuvette measurements in the Cary 100, compared to the concentrations determined via standards in the PlateReader AF2200 and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL cuvette	Calculated concentration in ng/µL standard	Deviation from cuvette measurement in %
100	104.1	Standard	Standard
80	82.7	78.96	- 4.7
60	61.9	59.50	- 4.0
50	51.7	Standard	Standard
25	25.6	Standard	Standard
10	10.1	10.25	+ 1.5
5	5.0	Standard	Standard

Table 9: 200 µl sample: Concentrations determined via cuvette measurements in the Cary 100, compared to the concentrations determined via standards in the PlateReader AF2200 and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL cuvette	Calculated concentration in ng/µL standard	Deviation from cuvette measurement in %
100	104.1	Standard	Standard
80	82.7	79.30	- 4.3
60	61.9	59.73	- 4.0
50	51.7	Standard	Standard
25	25.6	Standard	Standard
10	10.1	9.91	- 1.9
5	5.0	Standard	Standard

Table 10: 300 µl sample: Concentrations determined via cuvette measurements in the Cary 100, compared to the concentrations determined via standards in the PlateReader AF2200 and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL cuvette	Calculated concentration in ng/µL standard	Deviation from cuvette measurement in %
100	104.1	Standard	Standard
80	82.7	79.21	- 4.2
60	61.9	59.63	- 3.7
50	51.7	Standard	Standard
25	25.6	Standard	Standard
10	10.1	10.25	+ 1.5
5	5.0	Standard	Standard

Table 11: 100 µl sample: Concentrations determined via cuvette measurements in the Cary 100, compared to the concentrations determined via the sample-specific extinction factor in the PlateReader AF2200 and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL cuvette	Calculated concentration in ng/µL factor	Deviation from cuvette measurement in %
100	104.1	99.81	- 4.3
80	82.7	79.31	- 4.3
60	61.9	59.18	- 4.6
50	51.7	49.71	- 4.0
25	25.6	24.38	- 5.0
10	10.1	9.75	- 3.4
5	5.0	4.91	- 1.8

Table 12: 200 µl sample: Concentrations determined via cuvette measurements in the Cary 100, compared to the concentrations determined via the sample-specific extinction factor in the PlateReader AF2200 and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL cuvette	Calculated concentration in ng/µL factor	Deviation from cuvette measurement in %
100	104.1	101.89	- 2.2
80	82.7	80.72	- 2.5
60	61.9	60.12	- 3.0
50	51.7	50.25	- 2.9
25	25.6	24.76	- 3.4
10	10.1	9.58	- 5.4
5	5.0	4.73	- 5.7

Table 13: 300 µl sample: Concentrations determined via cuvette measurements in the Cary 100, compared to the concentrations determined via the sample-specific extinction factor in the PlateReader AF2200 and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL cuvette	Calculated concentration in ng/µL factor	Deviation from cuvette measurement in %
100	104.1	102.60	- 1.5
80	82.7	81.10	- 2.0
60	61.9	60.75	- 1.9
50	51.7	50.55	- 2.7
25	25.6	24.94	- 2.6
10	10.1	9.72	- 3.9
5	5.0	4.82	- 3.7

Table 14: 100 µl sample: concentrations determined via standards, compared to the concentrations determined via the sample specific extinction factor and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL factor	Calculated concentration in ng/µL standard	Deviation standard/ factor in %
100	99.81	Standard	Standard
80	79.31	79.31	- 0.4
60	59.18	59.18	+ 0.5
50	49.71	Standard	Standard
25	24.38	Standard	Standard
10	9.75	9.75	+ 5.1
5	4.91	Standard	Standard

Table 15: 200 µl sample: concentrations determined via standards, compared to the concentrations determined via the sample specific extinction factor and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL factor	Calculated concentration in ng/µL standard	Deviation standard/ factor in %
100	101.89	Standard	Standard
80	80.72	79.30	- 1.8
60	60.12	59.73	- 0.7
50	50.25	Standard	Standard
25	24.76	Standard	Standard
10	9.58	9.91	+ 3.4
5	4.73	Standard	Standard

Table 16: 300 µl sample: concentrations determined via standards, compared to the concentrations determined via the sample specific extinction factor and their respective deviations in percent

Prepared DNA concentration in ng/µL	Calculated concentration in ng/µL factor	Calculated concentration in ng/µL standard	Deviation standard/ factor in %
100	102.60	Standard	Standard
80	81.10	79.30	- 2.4
60	60.75	59.63	- 1.9
50	50.55	Standard	Standard
25	24.94	Standard	Standard
10	9.72	10.25	+ 5.5
5	4.82	Standard	Standard

Summary

As to seen from the results above (table 8-16), the factor based analysis of the data shows only minor differences to the calculation via standard curve. Same or even better are the results compared to the reference measurements performed in cuvettes. A summary of all results is in shown in table 17, exemplified for the results of 10, 60 und 80 ng/µL dsDNA.

Table 17: Summary of factor based and standard curve based measurements of dsDNA performed in the Eppendorf PlateReader AF2200 using the Eppendorf Microplate UV-VIS, compared to measurements performed in cuvettes using a spectrophotometer.

Prepared dsDNA concentration in	Results of cuvettes based measurements performed in a spectrophotometer (references)	Filling volume in the plate	Results of standard curve based measurements performed in the Microplate UV-VIS	Results of factor based measurements performed in the Microplate UV-VIS	Variance between standard curve and cuvettes based measurements	Variance between factor and cuvettes based measurements	Variance between factor and standard curve based measurements
ng/µL	ng/µl	µL	ng/µL	ng/µL	%	%	%
10	10.1	100	10.25	9.75	1.5	-3.4	5.1
60	61.9	100	59.5	59.18	-4.0	-4,6	0.5
80	82.7	100	78.96	79.31	-4.7	-4.3	-0.4
10	10.1	200	9.91	9.58	-1.9	-5.4	3.4
60	61.9	200	59.73	60.12	-4.0	-3.0	-0.7
80	82.7	200	79.3	80.72	-4.3	-2.5	-1.8
10	10.1	300	10.25	9.72	1.5	-3.9	5.5
60	61.9	300	59.63	60.75	-3.7	-1.9	-1.9
80	82.7	300	79.21	81.1	-4.2	-2.0	-2,4

Conclusion

As clearly shown by the comparative analyses of measurement results, factor-based determination of sample concentrations is possible in the Eppendorf PlateReader AF2200 when using the Eppendorf Microplate UV-VIS. The results are comparable amongst each other, as well as against reference measurements in standard cuvettes. The same is true for the comparison of factor-based results with analyses via standard curves, which represent the common method for concentration determination in a plate reader. Since filling height determines the path length, and therefore a fundamental parameter of concentration determination, highly reproducible dispensing of sample volumes is critical, as well as the use of plates with known and consistent well geometry. Splashes on the rim of the well or the formation of a pronounced meniscus (e.g. resulting from detergent-containing buffers) are to be avoided. Taken together, it is safe to state that factor-based calculation of the concentration in the Eppendorf PlateReader AF2200, in combination with the Eppendorf Microplate UV-VIS, represents a qualitative substitute to the common method of extrapolation from a standard curve. By eliminating the effort required to generate a standard curve, this method is very economical compared to the current common procedures, since both time and money are saved.

Literature

[1] Gallagher, Sean R. (2001), Quantification of DNA and RNA with Absorption and Fluorescence Spectroscopy, Current Protocols in Cell Biology, Appendix 3D

Ordering information

Description	Order no. international	Order no. North America
Eppendorf PlateReader AF2200 - 230V/50-60Hz, electrical plug Europe - 120V/50-60Hz, electrical plug North America	 6141 000.002 6141 000.010	 6141000010
Eppendorf µPlate G0.5 Eppendorf microvolume plate for Eppendorf PlateReader AF2200	6144 000.003	6144000003
µPlate G0.5 & PlateReader AF2200 (Bundle) Eppendorf microvolume plate and PlateReader AF2200 - 230V/50-60Hz, electrical plug Europe - 120V/50-60Hz, electrical plug North America	 6141 000.908 6141 000.909	 6141000922
UV/Vis filter slide for PlateReader AF2200 Pre-configured filter slide, optimized for applications in the UV and Vis ranges, 4 filters (260, 280, 340, 600nm)	6141 070.019	6141070019
Fluorescence filter slide for PlateReader AF2200 Pre-configured filter slide, optimized for the most common fluorescent dyes in the molecular and cell biology laboratory (360/465, 485/535, 485/595, 535/595)	6141 070.027	6141070027
Eppendorf filter slide case Storage case for up to two filter slides	6141 070.035	6141070035
UVette® 220 nm – 1,600 nm Plastic cuvette for measurements in the UV and Vis ranges, individually wrapped, certified PCR-clean and protein free, 80 pcs.	0030 106.300	952010051
UVette® routine pack 220 nm – 1,600 nm Plastic cuvette for measurements in the UV and Vis ranges, Eppendorf Quality, re-closable box, 200 pcs	0030 106.318	952010069
Eppendorf Microplate UV-VIS, 96/F Microplate UV-VIS, 96/F, clear wells, film bottom, PCR clean, 40 plates (4 bags of 10)	0030 741.048	0030741048

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