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Determination of nucleic acid concentrations using fluorescent dyes in the Eppendorf BioSpectrometer® fluorescence

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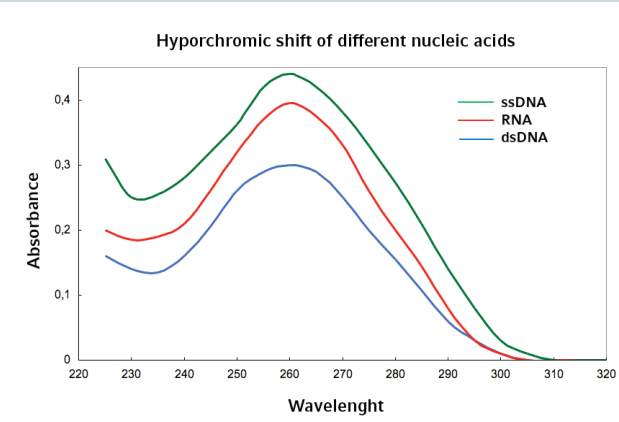
Abstract

Fluorescence allows more sensitive and more specific determination of nucleic acids than the conventional method of UV-VIS spectroscopy. The present article describes quantification of dsDNA using the fluorescent dye PicoGreen®, with special focus on the preparation of samples for measurement in the Eppendorf BioSpectrometer, as well as the details of programming. The methods PicoGreen and PicoGreen-"short" are compared experimentally. Even though the method PicoGreen-"short" was measured using only 2 standards, high analytical accuracy was observed.

Introduction

Challenges encountered during quantification of nucleic acids by UV-VIS spectroscopy

In general, nucleic acid concentrations are quantified by measurement of light absorption in UV-VIS spectrometers. Absorbance is measured at 260 nm, and the concentration is calculated using a specific conversion factor. Additional measurements at 230 nm, 280, 320 (or 340) nm can detect contaminations due to proteins or other organic materials as well as interfering particles. Unfortunately, photometric determination is not very sensitive. If a certain threshold is not reached, the amount of nucleic acid can generally not be distinguished from artifacts. In the case of high, as well as low, concentrations specific quantification is challenging when the sample is contaminated with other nucleic acids. All nucleic acids show an absorbance maximum at 260 nm and can therefore hardly be distinguished by photometric methods. The only difference is that single stranded nucleic acids show higher absorbance than double stranded nucleic acids (figure 1). This effect is also referred to as hyperchromicity [1]; however, naturally, it does not allow distinction between the molecules. On the contrary, the actual determination of the concentration of a nucleic acid is distorted by the presence of another.



Subtitle: Figure 1: Hyperchromicity for different nucleic acids

Overlapping absorbance maxima are also evident after plasmid DNA isolation performed without RNA digest. The proportion of RNA remaining in the eluate after classic plasmid preparation is around 90% [2]. Further challenges arising during this form of plasmid isolation include contaminations with phenol or proteins; therefore, accurate photometric determination of plasmid concentration cannot be performed.

Fluorescence measurements as an alternative

A further option to determine nucleic acids is indirect detection of the molecules via a fluorescent dye. This method is ideally suited to the specific determination of dsDNA, ssDNA and oligonucleotides, RNA as well as proteins. In addition to higher specificity, this method is also more sensitive than the conventional method of light absorption by a factor of 1,000-10,000. Table 1 shows an overview of some common fluorescent dyes as well as their respective detection limits. The fact that dyes bind specifically to the nucleic acid of interest is of great advantage; i.e. dsDNA can be detected specifically and at very low concentrations.

The dye PicoGreen by the company life technologies™, frequently used for quantification of dsDNA, provides a very high level of specificity. This dye is able to detect minute quantities of solubilized dsDNA. Even in the presence of equal amounts of RNA or single stranded DNA PicoGreen almost exclusively detects dsDNA. In contrast, other fluorescent dyes such as ethidium bromide also interact with RNA. In this case, the detection limit is not as low as that achieved with PicoGreen. The dye Hoechst H33258 is even more specific for dsDNA than PicoGreen; however, it is not as sensitive. Table 2 lists an overview of the methods used for detection of dsDNA.

Table 1: Examples of fluorescent dyes for the purpose of quantification of biomolecules. The data are from the manufacturers' manuals.

Dye	Target molecule	Measurement range
PicoGreen	dsDNA	0,025 – 1000 ng/mL
Hoechst H33258	dsDNA	0,1 – 10 µg/mL (dsDNA)
Ethidiumbromide	dsDNA, RNA	0,1 – 10 µg/mL (dsDNA)
RiboGreen®	RNA	1 – 1000 ng/mL
Oligreen®	ssDNA, Oligo-DNA	0,1 -1000 ng/mL
NanoOrange®	Protein	10 ng/mL-10 µg/mL

Table 2: Sensitivities of known detection methods for dsDNA and RNA, respectively

	Absorption	Fluorecence		
Method of detection	A260	Hoechst H33258	Ethidium-bromide	PicoGreen
DNA Measurement range	1–50 µg/mL	0,01–15 µg/mL	0,1–10 µg/mL	0,025–1000 ng/mL
RNA Measurement range	1–40 µg/mL	NA	1–40 µg/mL	Minimal sensitivity
Ratio DNA/ RNA	0.8	400	2.2	>100

What are the differences between fluorescence and absorbance measurements?

In principle, both detection methods work on the assumption that solubilized molecules interact with light at a specific wavelength. During absorption, light energy causes transition of the illuminated molecule's electrons to a higher energy level. During this process, light is absorbed. Following this absorption, the electrons thus excited will return to their original energy state. A photometer is capable of determining the ratio between the remaining amount of light and the initial amount of light used to excite the molecule via a detector, thus determining the absorbance. The concentration of molecules in a solution may be determined via a substance-specific constant, the coefficient of absorbance, and the optical path length (in the cuvette), by employing Lambert-Beer's, or Bouguer-Lambert-Beer's law, respectively: A=E*C*L (E=coefficient of absorbance, C= concentration, L=optical path length). In the case where sample concentration is to be determined via fluorescence, the sample is similarly illuminated by a specific wavelength. Again, light is first absorbed. If a fluorophore is excited, it, or its excited electrons, respectively, does not return directly to their original energy level. As a first step, the electron falls to a slightly lower energy level, followed by a second step during which it returns to its original state. In this case, fluorescent light is emitted (figure 2). The wavelength of the emitted light is always longer than the wavelength of the exciting light (figure 3)

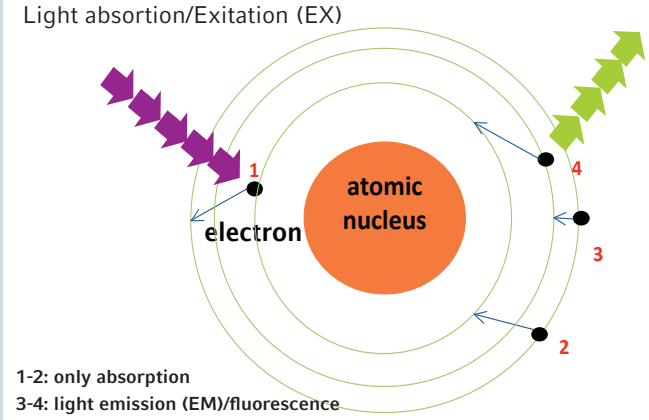


Figure 2: Principle of absorption and fluorescence measurements
1: by light absorption the electron is elevated to a higher energy level (absorbance measurement) and
2: electron returns to its original energy level
3: electron does not return directly to its original energy state; it moves to an intermediate level
4: electron jumps from the intermediate level to its original level (releasing fluorescent light)

One major difference to concentration determinations using absorbance measurements is that the above mentioned fluorophore such as PicoGreen is not measured directly, but rather it is employed for the indirect detection of molecules such as DNA. Calculations of sample concentrations rely on a previously generated standard curve. To this end, known nucleic acid concentrations (standards) are combined with the fluorescent dye, and relative fluorescence (RFU) is measured in a fluorimeter following interaction with the standards. This article will demonstrate how dsDNA may be detected in the Eppendorf BioSpectrometer fluorescence using a PicoGreen assay by the company life technologies (Quant-iT™ PicoGreen dsDNA Reagent and Kits). In addition to conventional determination using 5 standards as presented by the manufacturer, we will show that reliable determination is possible using only 2 standards. For other nucleic acid methods (RiboGreen, OliGreen), quantified in the Eppendorf BioSpectrometer, these "short protocols" (analysis using 2 standards: 0 and X ng/mL) are offered in addition to the conventional methods. One important advantage of the BioSpectrometer fluorescence is that it constitutes not only a fluorimeter, but rather a complete spectrometer.

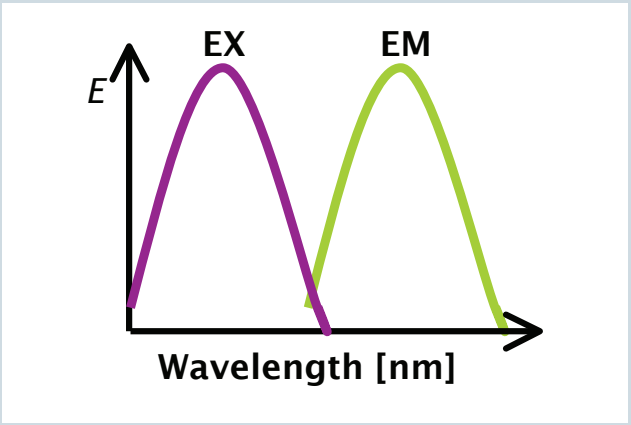


Figure 3: The light for excitation (EX) of the fluorophore has a shorter wavelength than the fluorescent light emitted (EM)

This advantage becomes apparent even during the preparation of standard solutions, as the standard stock solution requires spectrometric verification. Thus, all required measurements for analysis using the above mentioned kit may be prepared on one instrument.

Materials and experimental procedures

Materials: BioSpectrometer fluorescence, Quant-iT PicoGreen dsDNA Kit, VIS-Macro cuvettes, water (molecular grade), 2 x 50 mL test tubes, 1.5/2 mL Eppendorf Safe Lock Tubes™, Eppendorf MixMate®, Eppendorf UVette®, dsDNA stock solution (AppliChem®: A5187,1000), Eppendorf Research® pipettes

Generation of a standard curve using dsDNA-Standards
Preparation of the measurement solution was based on the user manual of the Quant-iT, PicoGreen dsDNA kit. Instead of 2 mL, only 1 mL measurement volume was used. This was possible since, due to the low height of the light ray of only 8.5 mm in the Eppendorf BioSpectrometer fluorescence, 1 mL is sufficient for measurement in macro cuvettes. When preparing a standard curve, careful attention needs to be paid to the standard concentration, which is to correspond to the final volume of the entire preparation. This fact needs to be taken into consideration during subsequent calculations of sample concentrations. For this reason, samples and standards need to be prepared in the same manner. Since the measurements are considerably more sensitive than absorbance measurements, as mentioned above, all instruments, especially pipettes, need to be in a technically impeccable state. It is further recommended to acclimatize all kit components to room temperature for 2 h prior to use. First, the assay buffer is prepared. For this purpose, 1 mL of the 20x TE buffer provided in the kit is diluted in 19 mL water (molecular grade) in a 50 mL test tube. The reagent solution is provided as a concentrate in 100 µL aliquots. 50 µL are removed and diluted 200-fold in the freshly prepared assay buffer to 10 mL (0.05 mL reagent solution + 9.95 mL assay buffer). This amount is sufficient for approximately 20 measurements incl. samples and standards. The concentration of the DNA stock solution provided in the kit is 100 ng/µL. This is first diluted 50-fold in assay buffer. The final concentration of this primary DNA solution, the starting solution for generation of the standard curve, should measure 2 ng/µL, or 2000 ng/mL, respectively. This primary solution should be verified photometrically. A dsDNA solution of 2 ng/µL corresponds to an optical density of 0.04 at 260 nm, measured in a cuvette with a light path of 10 mm. Since the Eppendorf BioSpectrometer fluorescence is a complete UV-VIS spectrometer, this instrument is fully capable of performing the photometric verification of the primary solution, as well as the subsequent fluorime-tric measurements. Eppendorf UVettes may be used for the photometric measurements. The freshly prepared 1 x TE buffer serves as the blank solution. For the individual photometric measurements, the method dsDNA is selected. In order to test whether the primary solution is free from contamination, the "scan" should be activated prior to measurement in the area "check parameters", as well as the ratio 260/230 and the background correction at 320 nm or 340 nm, respectively (figure 4A and 4B).

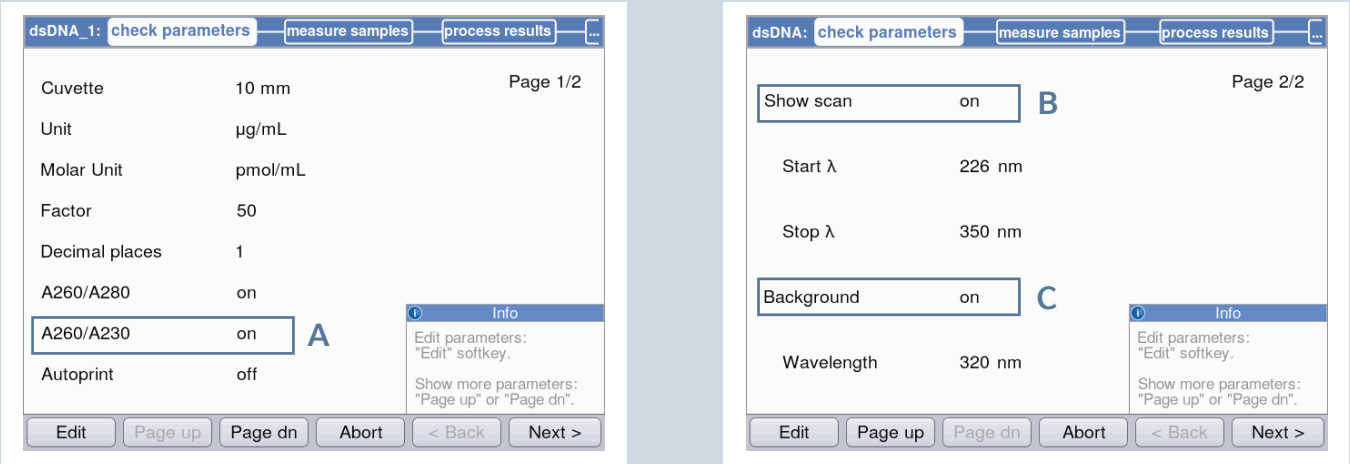


Figure 4: Settings for measurement of the primary solution in the Eppendorf Biospectrometer fluorescence
A: A260/A230
B: Activation of "scan"
C: Activation of background

Figure 5 shows that the primary solution for the standard curve contains the concentration stated by the manufacturer. In case the expected concentration cannot be found, the values for the standard curve may be adjusted accordingly. For example, if a dsDNA concentration is measured at only 1.6 µg/mL, the standard concentrations may be generated as follows (please also refer to table 3): 800 ng/mL, 80 ng/mL, 8 ng/mL, 0.8 ng/mL and 0 ng/mL.

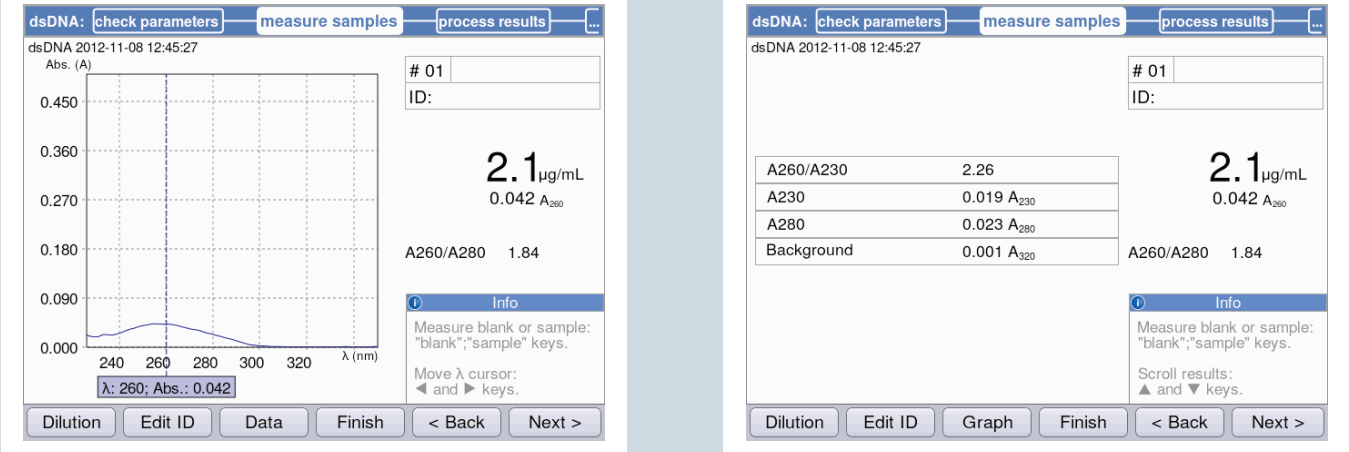


Figure 5: Verification of the concentration of the primary solution for the standard concentrations.

Following verification of the dsDNA primary solution the subsequent solutions for the standard measurements can be generated. As described above, the measurement volume is only 1 mL instead of the 2 mL recommended by the kit manufacturer, which translates to a considerable saving in reagents. Ideally, 2-3 identical replicates are prepared for each dilution step. Table 3 shows the pipetting scheme for each dilution.

Table 3: Pipetting scheme for the respective standard concentrations

Dilution step	Assay buffer volume [µL]	Volume of dsDNA primary solution [µL]	Volume of diluted reagent solution [µL]	Final dsDNA concentration in total volume [ng/mL]	Standard number
1	0	500	500	1000	5
2	450	50	500	100	4
3	495	5	500	10	3
4	499.5* (495)	0.5 (5 µL 1:10)	500	1	2
5	500	0	500	0	1

*The primary solution was to be diluted 1:10 (e.g. 5 µL DNA solution plus 45 µL assay buffer). From this diluted solution, 5 µL are diluted in 495 µL assay buffer).

It is imperative to treat the samples in the same manner as the standards. In this case it means that 500 µL sample (if possible, in 1 x TE buffer) are to be mixed with 500 µL reagent solution. If samples need to be diluted due to a high signal, the dilution should always be performed using fresh 1 x TE buffer. If the dilution is programmed into the instrument, the primary concentration is automatically calculated and displayed. It is to be noted that the standard containing 0 ng/mL simultaneously serves as the blank. In addition, the standards should be prepared in an Eppendorf Safe-Lock tube and mixed thoroughly directly prior to measurement, for example in an Eppendorf MixMate®.

Programming and measurement of the standard curve:
The method PicoGreen is used for determination of dsDNA.
The number of standards, as well as the standard concentration, may be programmed in the area “check parameters”.

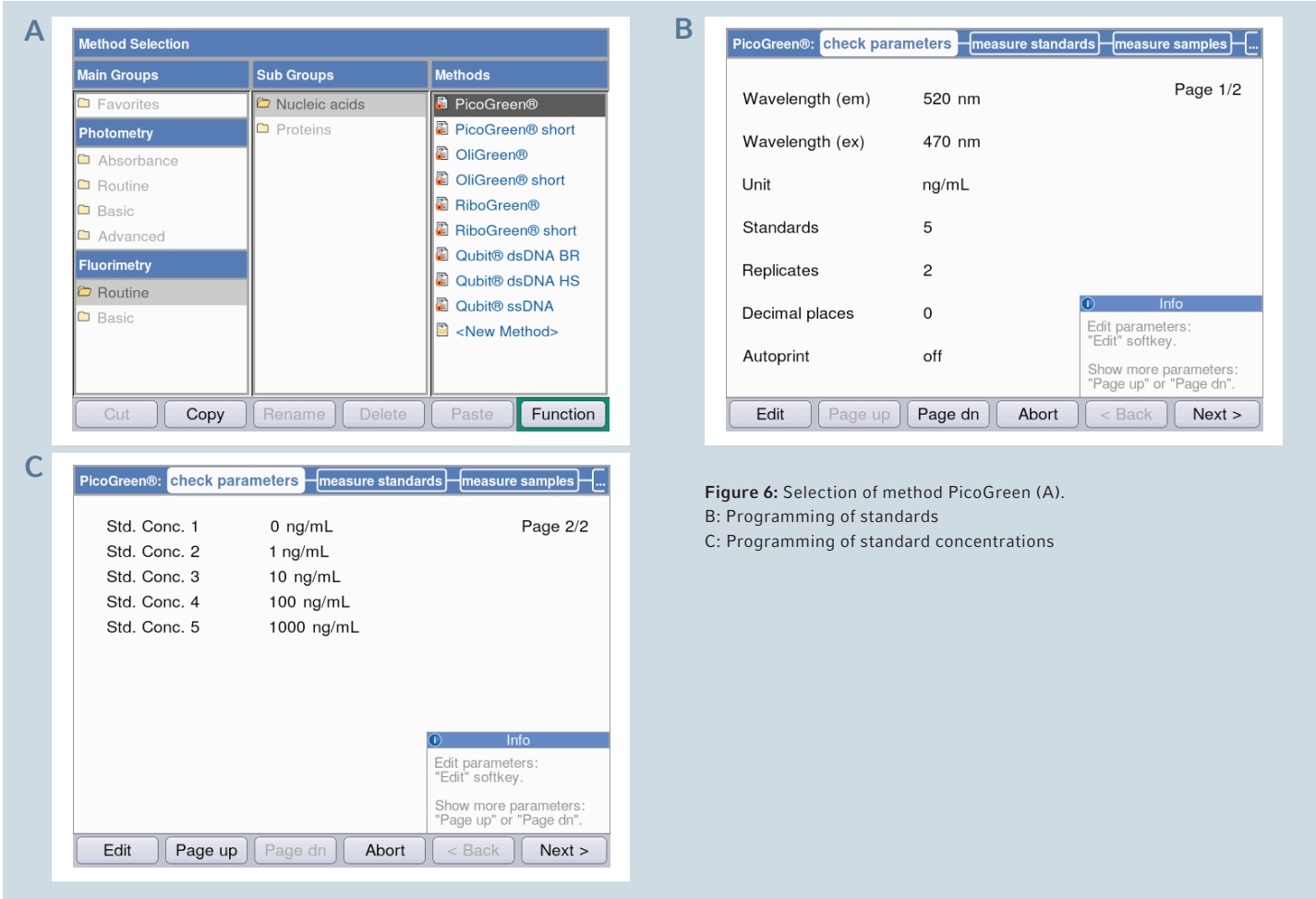


Figure 6: Selection of method PicoGreen (A).
B: Programming of standards
C: Programming of standard concentrations

All parameters are pre-programmed in accordance with the information provided in the protocol for the Quant-iT PicoGreen dsDNA Kit by life technologies. By pressing the softkey "next", one will arrive at the area "measure standards". Due to photobleaching, i.e. diminishing of the fluorescence signal in the sample over time, all standards and samples should be measured as quickly in succession as possible. During measurement, the display may be alternated between table and graphic formats (figure 7). The linear progression of the curve, and an R^2 (coefficient of determination), which may not be less than 0.95, are critical for analysis. In case the R^2 value does fall short of this value, the instrument will issue a warning.

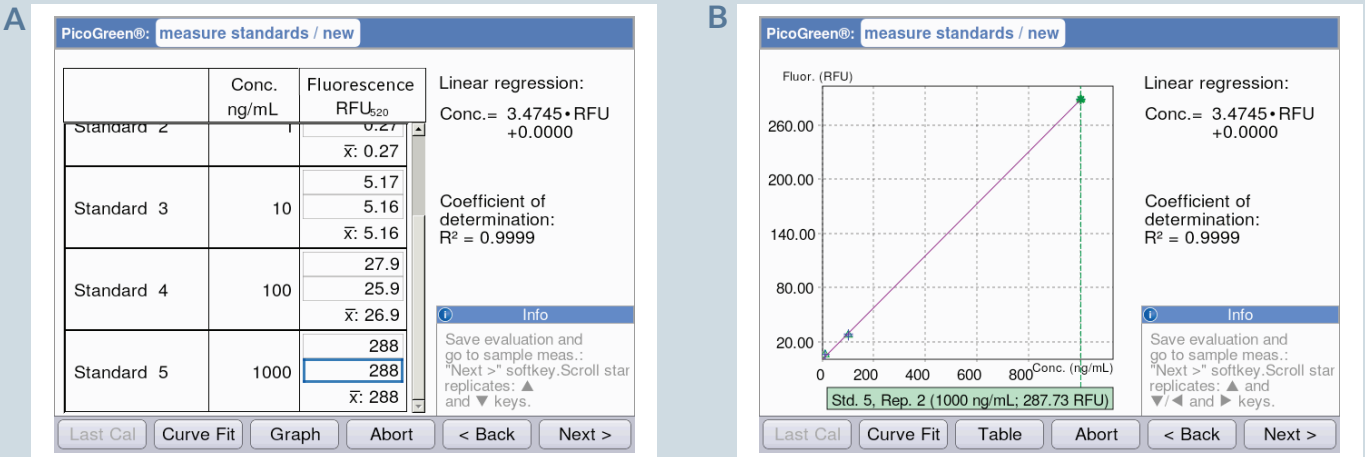


Figure 7: Generation of the standard curve with defined dsDNA standard concentrations.
A: Table format / B: Graphic format

Use of the PicoGreen "short" method:

For measurements using standard curves which are strictly linear, such as determination of dsDNA using PicoGreen, it is possible to reduce the number of standards. For this purpose, the "short" methods are offered in the Eppendorf BioSpectrometer fluorescence: only a zero standard and one additional standard of a defined concentration are measured. The second standard has to fall inside the linear range. In this case, analysis is performed via linear interpolation, as linear regression with only 2 standards is not possible. 0 and 500 ng/mL are pre-programmed for the PicoGreen- "short" method (figure 8). Programming of the "short" method is simple and fast. The results section will demonstrate the fact that sample quantifications performed with such 2 point calibrations will yield similarly accurate results as those sample quantifications achieved with conventional analysis using 5 standards.

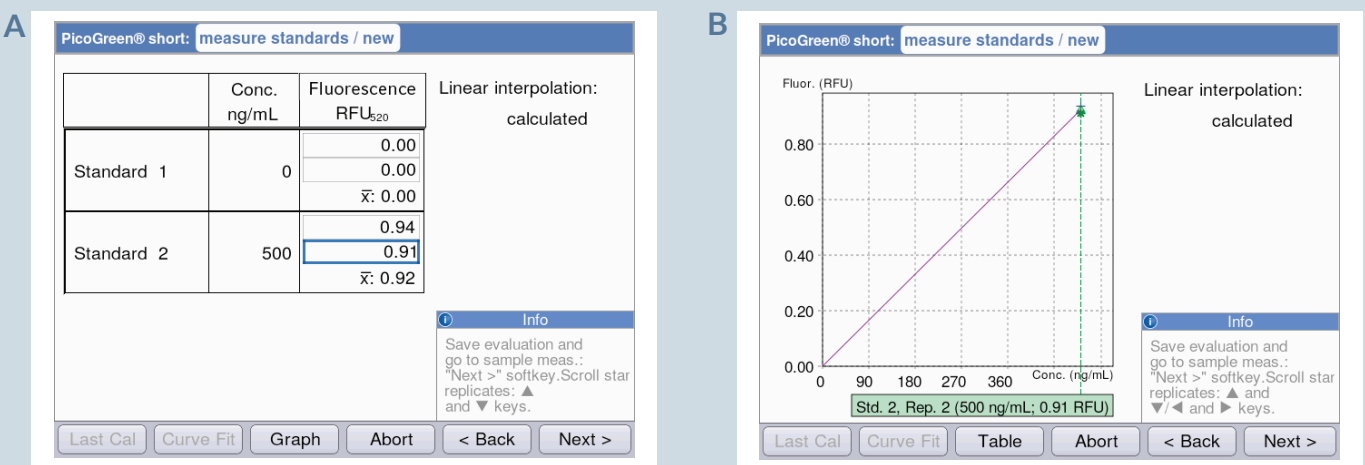


Figure 8: Generation of a standard curve using the PicoGreen "short" method
A: Table format display / B: Graphic format display

Quantification of RNA, ssDNA and Oligo-DNA
In addition to the PicoGreen method, other methods for determination of RNA via RiboGreen and ssDNA, or oligo-DNA, respectively, via OliGreen, are pre-programmed. The methods are guided by the kits by Life Technologies: Quant-iT" RiboGreen RNA Reagent and Kit (RNA)" and Quant-iT "OliGreen ssDNA Reagent and Kit", respectively. The quantification using these methods is analogous to the analysis using PicoGreen. This is also the case for the "short" methods which are equally pre-programmed. For the RiboGreen kit it may be advantageous to employ non-linear regression for the standard curve instead of the linear regression recommended in the kit manual. In the Eppendorf BioSpectrometer fluorescence, the regression may be adapted during the process of standard measurements, while generating the standard curve. In any case, the regression which optimally fits the shape of the standard curve should be used. The best fit is determined by the coefficient of determination R^2 (min.: 0.95/ max.: 1).

Experimental procedures for fluorimetric measurements using the Eppendorf BioSpectrometer fluorescence
Fluorophores such as the dyes mentioned above are very light sensitive. Their fluorescent properties will decrease with time due to increasing light influence ("photobleaching"). For this reason, cool and dark storage conditions are recommended for the respective reagents. The effect of photobleaching is also noticeable during the measurement process; therefore the samples should be measured in quick succession.
Fluorophore stability varies greatly; PicoGreen is relatively stable, whereas RiboGreen quickly loses its fluorescent properties under the influence of light. A fresh standard curve should be generated for each quantification experiment since, due to the sensitivity of the methods, 100% reproducible conditions are difficult to achieve under the influence of changing light intensity or temperature. For these reasons it is recommended to prepare samples of one series under homogeneous light conditions.
Since the preparations for the actual measurements are quite involved compared to quantification using UV-VIS spectroscopy, it is useful to collect samples prior to the start of a series of measurements. This approach also helps save reagents.
For all measurements, the zero standard simultaneously serves as the blank. As mentioned above, it is important to verify that the standard curve corresponds to the pre-programmed regression analysis (curve fit). Subsequent adjustment is possible at all times during the generation of the standard curve. The analysis method may be changed at any time during measurement by employing the function "curve fit".
It would be more accurate to generate the standard curve by duplicate or triplicate measurements for each standard, thus facilitating detection of an abnormal value. During this process, measurement of each standard may be repeated. During measurement, careful attention is to be paid to the RFU values; values below 1 should not be considered. It is further critical to ensure proper mixing of the samples, in particular the standards, prior to measurement.

Results and discussion

The methods "PicoGreen" and "PicoGreen-"short" are to be compared. The pre-programmed "short" methods are characterized by the advantages that (a) standard measurement is completed noticeably faster and (b) considerable amounts of reagents may be saved. Only two standards are measured, e.g. 0 ng/mL and 500 ng/mL for PicoGreen-"short" in the BioSpectrometer fluorescence. Again, the zero standard doubles as the blank value. Since the standard curve for PicoGreen analysis using 5 standards is strictly linear, as shown in figure 7b, one may work on the assumption that 2 standards suffice for analysis. For the purpose of direct comparison between both methods two standard curves were generated: one with 2 standards (0 and 500 ng/mL) and one with 5 standards (0, 1, 10, 100 and 1000 ng/mL), followed by quantification of samples of the concentrations 10, 100 and 1000 ng/mL using both standard curves. The results are shown in figure 9. The screenshots 9 A-C are based on analysis using 5 standards, whereas 9 D-F show results based on analysis using 2 standards.

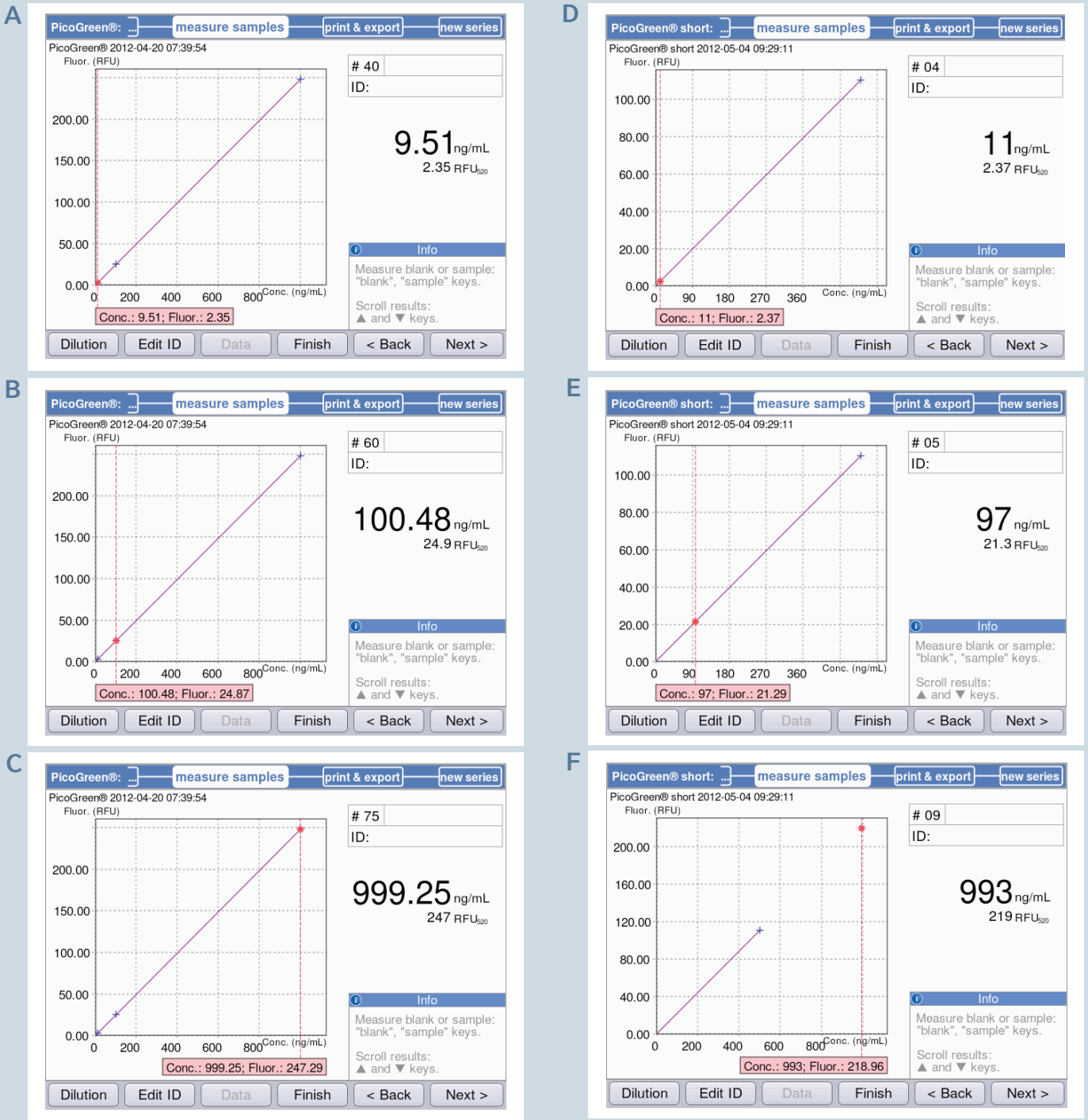


Figure 9: Quantification of dsDNA samples (10, 100 and 1000 ng/mL) using the methods PicoGreen and PicoGreen "short".
A-C: Method PicoGreen
D-F: Method PicoGreen -„short"

Both results are comparable, as shown in figure 9. Analysis using 5 standards is slightly more accurate in this example; however, the "short" method is sufficient for quick verification of samples. This fact holds true when the samples are measured in a reference instrument, based on the method PicoGreen- "short", using 2-point calibration (table 4). The results fall within the expected range and deviate only minimally.

Table 4: Comparison of dsDNA quantification using the method "PicoGreen short" on the BioSpectrometer fluorescence and a reference instrument

Nominal concentration [ng/mL]	Results in reference instrument (2 Standards) [ng/mL]	Deviation from nominal value [ng/mL] / [%]	Result in the Biospectrometer using PicoGreen-"short" (2 Standards) [ng/mL]	Deviation from nominal value [ng/mL] / [%]	Difference between BioSpectrometer and reference instrument [%]
10	11.2	1.2 / 12	11	1 / 10	2.4
100	97.2	-2.8 / 2.8	97	-3 / 3	0.8
1000	993.1	-7 / 0.7	993	-7 / 0.7	0.0

As shown in table 4, and also in figure 9, the method PicoGreen "short" is able to deliver sufficiently accurate DNA quantification. The results deviate only minimally from the nominal value, and the deviations from the reference instrument are small. One major advantage of the Eppendorf BioSpectrometer is the fact that when a standard curve is used, the results of the samples are always displayed within the graph, in relation to the standard curve. This feature allows instant verification that the sample concentration falls within the range of the standard curve generated. Thus, this feature quickly eliminates the risk of false estimation of results. All measurements depicted above were performed in macro-cuvettes made from PMMA (poly-methylmethacrylate) in a 1 mL volume. However, the Eppendorf BioSpectrometer fluorescence may be used in combination with all commonly available cuvettes. For the purpose of fluorescence measurements, macro-cuvettes made from quartz, half-micro-cuvettes made from PMMA, ultra-micro-cuvettes made from quartz, or the Eppendorf UVette® may be used. Use of the UVette could further reduce the volume considerably, as 60 µL are sufficient for measurement. It should be noted, however, that the consequence of a reduced reagent volume is a reduced fluorescent signal. Samples at the lower end of the curve could therefore be subject to inaccuracies during quantification of low concentrations. Nucleic acid quantification via fluorescence allows measurement of considerably lower concentrations of dsDNA than the standard method of UV-VIS spectroscopy. The ability of the Eppendorf BioSpectrometer fluorescence to quantify dsDNA via fluorescence as well as via absorbance results in a measurement range which is unique on the market. This range is further extended when considering microvolume cuvettes, such as the Eppendorf µCuvette™ G1.0 [4], resulting in a theoretical range between 1 ng/ml, up to 1,500,000 ng/mL (= 1.5 mg/mL).

Conclusion

The Eppendorf BioSpectrometer fluorescence is a complete UV-VIS spectrometer with integrated fluorimeter. All methods implemented on the Eppendorf BioSpectrometer basic are also available. The integrated fluorimeter enables additional standard methods for quantification of nucleic acids and proteins by fluorescence. The pre-programmed methods are based on the Quant-iT kits by the company life technologies. In addition to analysis using 5 standards, as recommended in the kits, "short" methods are offered for all nucleic acids, which allow analysis using only 2 standards. The example of PicoGreen demonstrated that analysis using 2 standards is possible without compromising accuracy. The results were validated in a reference instrument. The "short" methods, in combination with volume reduction achieved by using the Eppendorf UVette, result in considerably reduced consumption of reagents. The combination of the Eppendorf µCuvette G1.0 and the Eppendorf BioSpectrometer fluorescence allows quantification of dsDNA across the broad range between 1 and 1,500,000 ng/mL (= 1.5 mg/mL).

Literature

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Ordering information

Product	Order no. International	Order no. North America
Eppendorf BioSpectrometer® fluorescence 230 V/50-60 Hz, electric connection Europe, additional electric connections available	6137 000.006	
120 V/50-60 Hz, electric connection North America	6137000.014	6137000014
Eppendorf BioSpectrometer® fluorescence set of reference filters Set of filters for verification of photometric accuracy and wavelength accuracy (in accordance with NIST) as well as for the verification of fluorimetric precision (random measurement deviation) and linearity	6137 928.009	6137928009
Eppendorf µCuvette™ G1.0 – Eppendorf micro volume –measurement cell for Eppendorf BioPhotometer and BioSpectrometer	6138 000.018	6138000018
Thermo printer DPU 414 incl. adapter and printer cable		
230 V, EU	6131 011.006	
115 V/100 V, USA	6131 010.000	952010140
JP 230 V	6131 012.002	
Thermo paper 5 rolls	0013 021.566	952010409
UVette® 220 nm – 1 600 nm Original Eppendorf plastic cuvette individually wrapped, certified RNase-, DNA- and protein-free 80 pcs	0030 106.300	952010051
UVette® routine pack 220 nm – 1 600 nm Eppendorf quality purity, re-sealable box, 200 pcs.	0030 106.318	952010069
Cuvette rack for 16 cuvettes	4308 078.006	940001102

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