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Troubleshooting Guide for the Measurement of Nucleic Acids with Eppendorf BioPhotometer® D30 and Eppendorf BioSpectrometer®

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Introduction

This Troubleshooting Guide will help you to achieve reliable results using devices of the Eppendorf photometer family (fig. 1) with focus on nucleic acid quantification. Therefore, the critical factors for attaining a precise measurement are summarized and recommendations are given on how to solve problems that might be encountered.

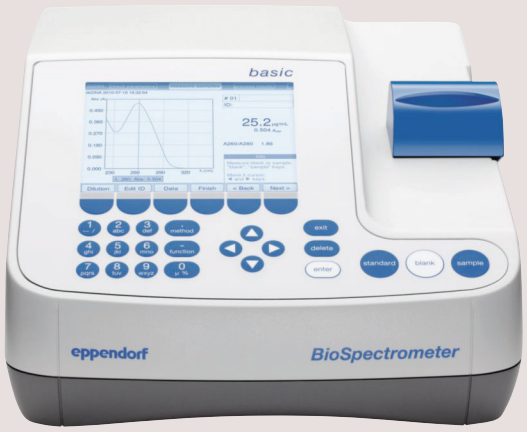


Figure 1: Eppendorf BioPhotometer D30 – one member of the Eppendorf Photometer family

Fundamentals of photometric measurements - Lambert-Beer Law

To calculate the concentration of the liquid sample in a cuvette, first the transmission (T) is measured by the photometer, while calculating the ratio of outgoing (I_1) and ingoing light (I_0) (fig. 2). The negative common logarithm of the transmission is the absorbance (A) value. The measured absorbance in a photometer depends on the optical path length, the concentration and a sample specific factor, the absorbance coefficient. This dependency is also called the Lambert-Beer-Law.

As shown in fig. 2D the concentration of the sample can be calculated via transforming the Lambert-Beer Law. This can be done very easily if you look on the physical constants and parameters that are described in the Lambert-Beer Law. In table 1 all the important parameters and the corresponding SI-units are listed. The concentration can only be calculated correctly if the SI-units of the absorbance coefficient fit to the SI-units of the concentration.

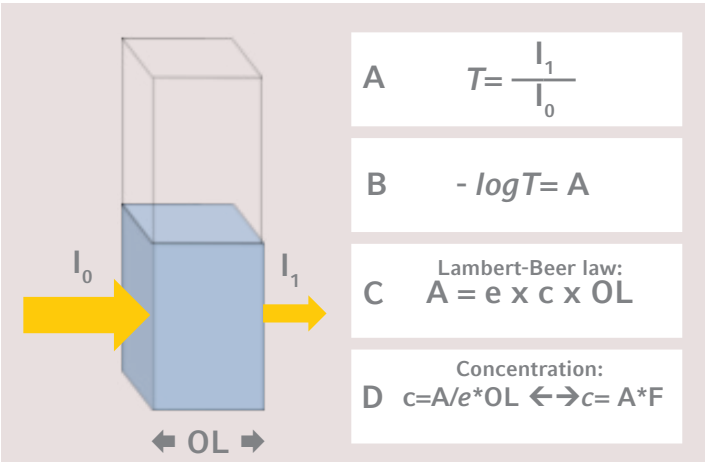


Figure 2:
Measuring of light absorbance in a photometer with a liquid sample (Yellow arrow: Light beam of the photometer)
A: Transmission
B: Calculation of absorbance
C: Lambert-Beer Law: A=absorbance, e= absorbance coefficient, c= sample concentration, OL=optical path length
D: Calculation of sample concentration via Lambert-Beer Law: F=1/e

Table 1: Physical constants/parameters and corresponding SI-units of the Lambert-Beer law.

Absorbance coefficient	$(Mol/L)^{-1} * cm^{-1}$, $(g/L)^{-1} * cm^{-1}$, $(mg/mL)^{-1} * cm^{-1}$, $(\mu g/mL)^{-1} * cm^{-1}$
Concentration	mol/L, mg/mL, µg/mL, g/L
Optical Path Length (OL)	mol/L, mg/mL, µg/mL, g/L

Calculation example for dsDNA:
If the measurement is carried out in a cuvette with an optical path length (OL) of 1 cm, the calculation of the concentration can be carried out directly via the formula c= A * F (fig. 2D). The factor F is in this case the reciprocal value of the absorbance coefficient. The absorbance coefficient is not only specific for specific samples but also for a certain wavelength, e.g. the coefficient for nucleic acids is only valid at 260 nm.
In table 2 the absorbance coefficients are listed for some nucleic acid molecules at 260 nm and the corresponding sample specific factor:

Table 2: Factors and absorbance coefficients for nucleic acids

Molecule	Absorbance coefficient 260 nm	Factor (in 1 cm cuvette)
dsDNA	0.020 $(\mu g/mL)^{-1} * cm^{-1}$	50 µg/mL
RNA	0.025 $(\mu g/mL)^{-1} * cm^{-1}$	40 µg/mL
ssDNA	0.027 $(\mu g/mL)^{-1} * cm^{-1}$	37 µg/mL

If a dsDNA sample shows an absorbance of 1 in the photometer measured in a cuvette with 1 cm optical path length the concentration of the sample is 50 µg/mL:

Lambert-Beer:
A=e*c*OL <> c=1/(e*OL)*A <> c=1/e *1/OL*A<> c=F*1/OL*A
<> c= 50 µg*cm/mL* 1/1 cm *1

= 50 µg/mL * 1 = 50 µg/mL

Lambert-Beer law:
A = e * c * OL
c = 1/(e*OL) * A
c = 1/e * 1/OL * A (1/e = F)
c = F * 1/OL * A (F = 50 (µg*cm)/mL, OL = 1 cm A = 1)
c = 50 (µg*cm)/mL * 1/1 cm * 1
c = 50 µg/mL

Analyze the measuring results of a nucleic acid sample with the Eppendorf BioPhotometer® D30 and Eppendorf BioSpectrometer®

Before you start measuring, it is important that the correct parameters for the measurement are set. With the Eppendorf BioPhotometer and BioSpectrometer these parameters can be edited directly before the measurement (fig.3)

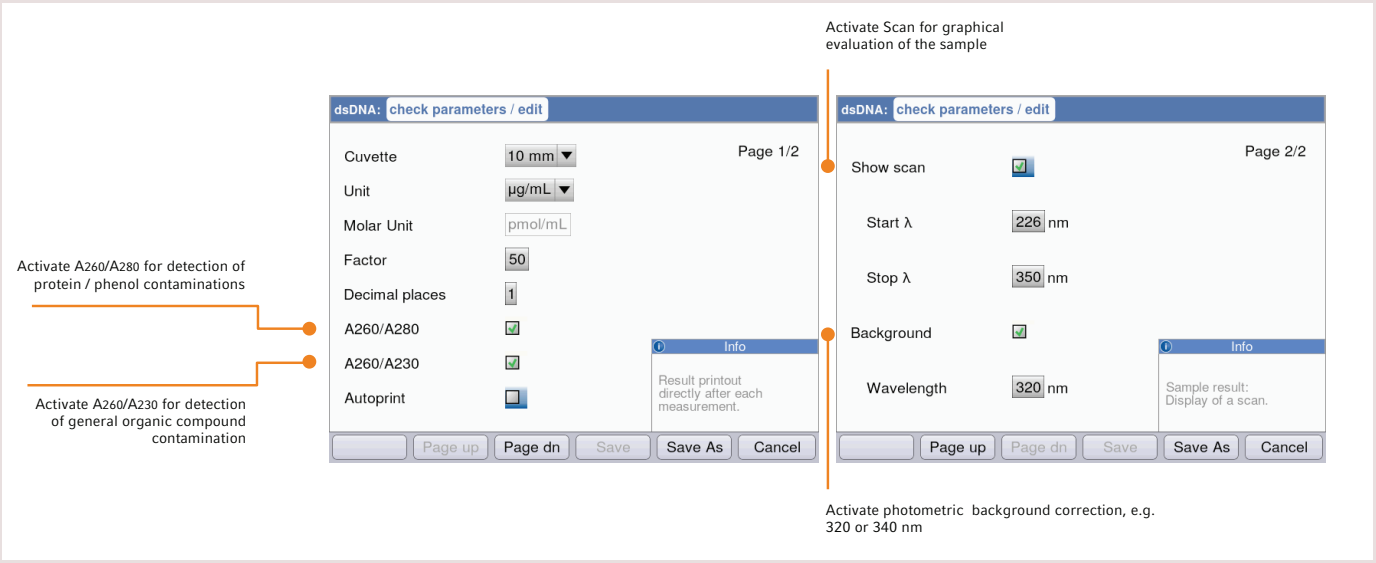


Figure 3: Parameter settings for nucleic acid quantification – Example BioSpectrometer

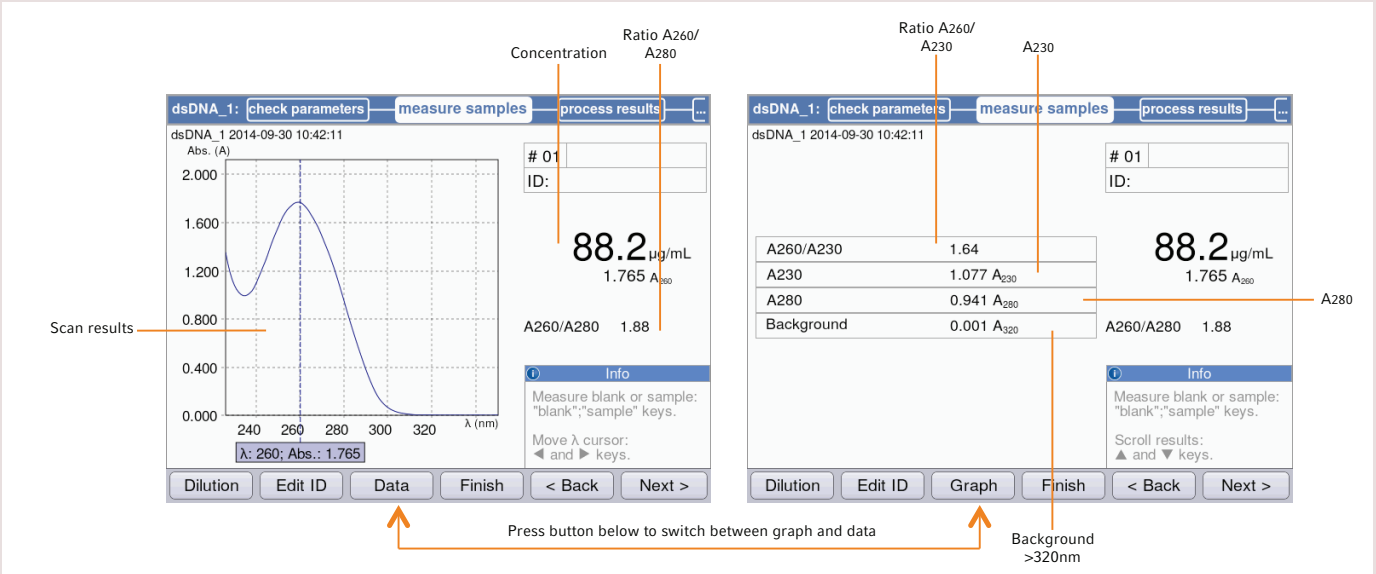


Figure 4: Measuring result of a nucleic acid sample with the BioPhotometer D30 or BioSpectrometer: You can switch between a graphical and table view of the result. In the table view more details about the sample quality is displayed.

Evaluating the measuring results

1) Absorbance reading at 260 nm (A260) – Nucleic acid sample concentration

Check	Problem	Solution
Is the sample in the linear absorbance measuring range of 0.05 – 2 A?* **Corresponding nucleic acid concentrations:** 1 cm (10 mm): UVette® 10 mm path length **dsDNA:** 2.5 - 100 µg/mL **RNA:** 2 - 80 µg/mL **ssDNA:** 1.85 - 74 µg/mL 0.2 cm (2mm): UVette 2 mm path length **dsDNA:** 12.5 - 500 µg/mL **RNA:** 10 - 400 µg/mL **ssDNA:** 9.25 - 370 µg/mL 0.1 cm (1 mm): Eppendorf µCuvette® G1.0 with 1 mm path length **dsDNA:** 25 - 1000 µg/mL **RNA:** 20 - 800 µg/mL **ssDNA:** 18.5 - 740 µg/mL	**Absorbance >2:** Above an absorbance of 2 the displayed result might not be in the linear range anymore due to stray light effects of the sample.	1) Dilute sample until the absorbance of the sample lies in the linear range 0.05 - 2. 2) Use cuvette with shorter optical path length: At high concentrations you can also turn the UVette using the 2 mm path length (fig. 5) or use the µCuvette G1.0 with 1 mm path length (fig. 6). The principle of decreasing the measuring path length is described in figure 7.
	Absorbance <0.05: With extinctions of less than 0.05, all sources of error, regardless of the device (e.g. imprecisions by pipetting, particulate matter etc.), become too significant to allow exact measurements.	Concentrate your sample, or if it is a diluted sample, decrease the dilution of your sample. For very low concentrated samples it is also possible to evaluate the sample via fluorescence, e.g. with the Eppendorf BioSpectrometer fluorescence [1].

*Valid for the measuring range of the BioPhotometer D30 and the BioSpectrometer

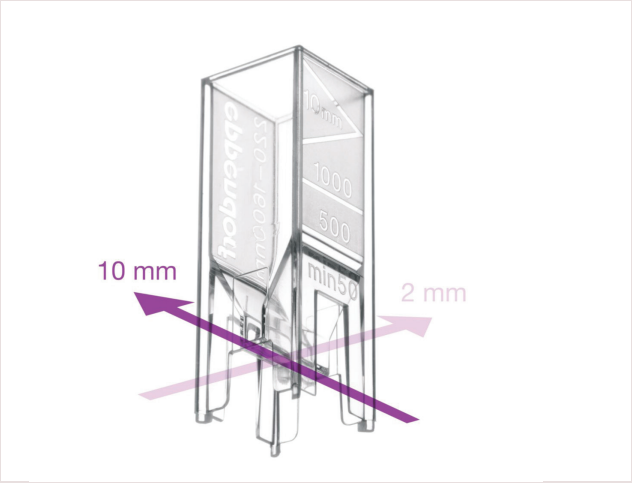


Figure 5: UVette with two optical path lengths: 10 mm and 2 mm

Figure 6: Eppendorf µCuvette G1.0 1 mm optical path length (orange circle)

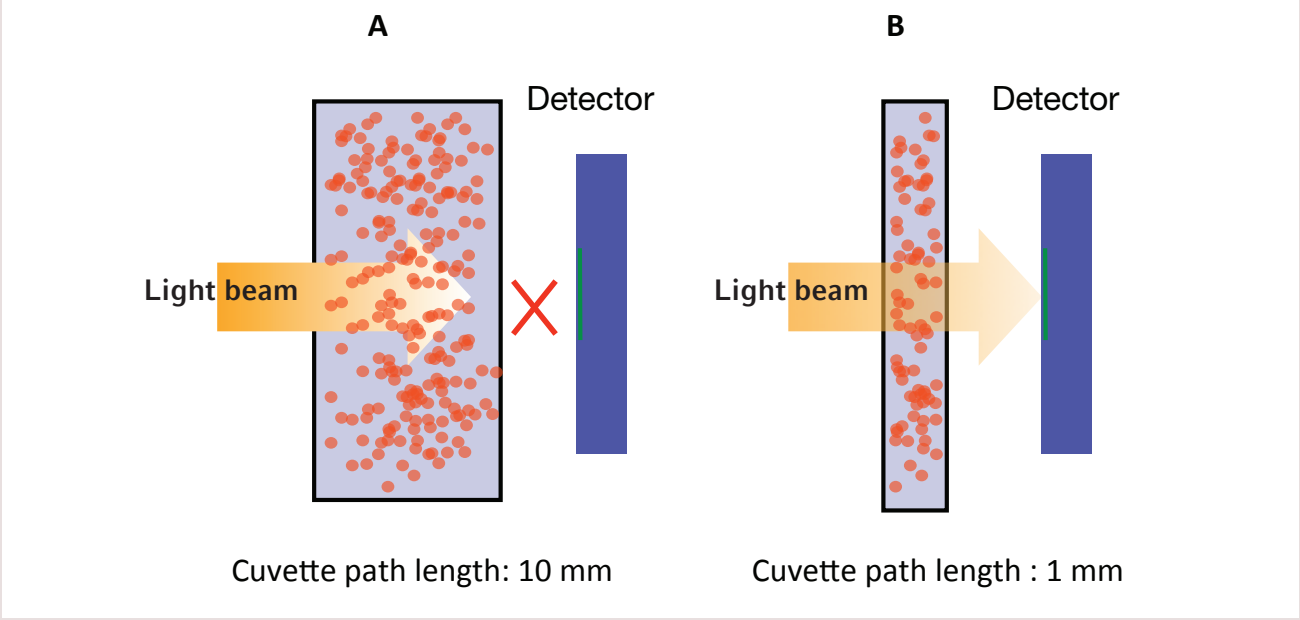


Figure 7: Smaller path lengths for higher concentrations

2) Check absorbance reading at 280 nm – Nucleic acid sample quality and possible contamination with protein or phenols/ Determination of the purity ratios A260/A280

Check	Problem	Solution
By measuring the absorbance at 260 nm and 280 nm the ratio A260/A280 can be determined: Ratio for pure nucleic acid samples should be: **1.8 - 2.0**	**A260/A280 <1.8:** Since proteins or phenols show a high absorbance in this range, a too low ratio could indicate a contamination of the sample. For example, a ratio of 1.5 corresponds to a 50 % protein/DNA ratio.	Purify your sample if possible! Try quantification with fluorescence, if possible [1].

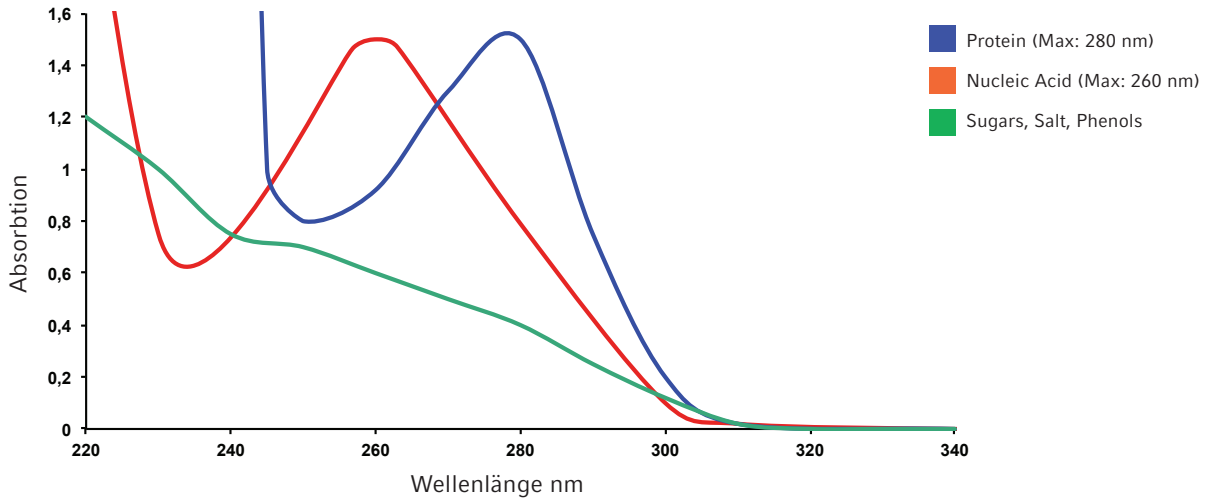
3) Check absorbance reading at 230 nm – Nucleic acid sample quality and possible contamination with any organic compounds/ Determination of the purity ratios A260/A230

Check	Problem	Solution
By measuring the absorbance at 260 nm and 230 nm the ratio A260/A230 can be determined: Ratio should be: **>2.0**	**A260/A230 <2.0:** The ratio value should be higher than 2.0 for pure DNA and RNA. Values less than 2.0 indicate contamination by sugars, salts or organic solvents. A contamination by proteins would also have an influence on the 260/230 ratio.	Purify your sample if possible. Try quantification with fluorescence, if possible [1].

4) Check absorbance reading >320 nm – Nucleic acid sample quality and photometric background

Check	Problem	Solution
Above 320 nm there is no absorbance of nucleic acid or from possible contamination by organic compounds (e.g. proteins). **A320=0.0**	**A320 nm > 0.0** Pure nucleic acid sample should show no absorbance in this range. If there is absorbance, there is photometric background in the sample. Possible reason for measured photometric background: > Turbidity: Microbial growth, magnetic beads, precipitation > Air bubbles > Optical parts of the cuvette are dirty > No liquid column, because volume is too small (µCuvette) > Not enough liquid in the cuvettes (Example UVette: > 50µL)	If the photometric background is <0.03 you can activate the background correction of your photometer: all measured values are reduced by the absorbance value obtained at 320 nm. If the background is >0.03 you can (depending on the reason for background absorbance): > Purify the sample > Clean optical parts of the cuvettes if necessary > Remove air bubbles > Increase sample volume (UVette or µCuvette)

In fig. 8 it is explained how certain types of contamination could influence the measuring result of a nucleic acid sample at 260 nm.



Purity Ratios:
a) A260/A280 = 1.8 – 2.0
b) A260/A230 > 2.0

low a) Contamination by proteins
low b) Contamination by organic compounds including proteins

Figure 8: Influence on the measuring result at 260 nm by protein or other organic compounds

Error message in the results display

Common reasons for error messages

Problem	Solution
The measured absorption is higher than 3.0: Example fig. 9B	> Dilute your sample. > Check whether your cuvette is transparent at a light beam height of 8.5 mm. All Eppendorf cuvettes meet this requirement. > Clean the cuvette shaft. > Check whether the cuvette is inserted in the correct direction (measuring window in the direction of the light path). > Check if the cuvette is suitable for UV measurement.
The ratio cannot be calculated because the absorption of one of the used values is 0.0 or higher than 3.0: Example fig. 9A	> Purify your sample. > Some nucleic acid purification kits contain elution buffers that show significant high absorbance at 230 nm. In this case you cannot expect a reasonable A260/A230. If you need a reasonable ratio change the elution buffer (TE-buffer, water).

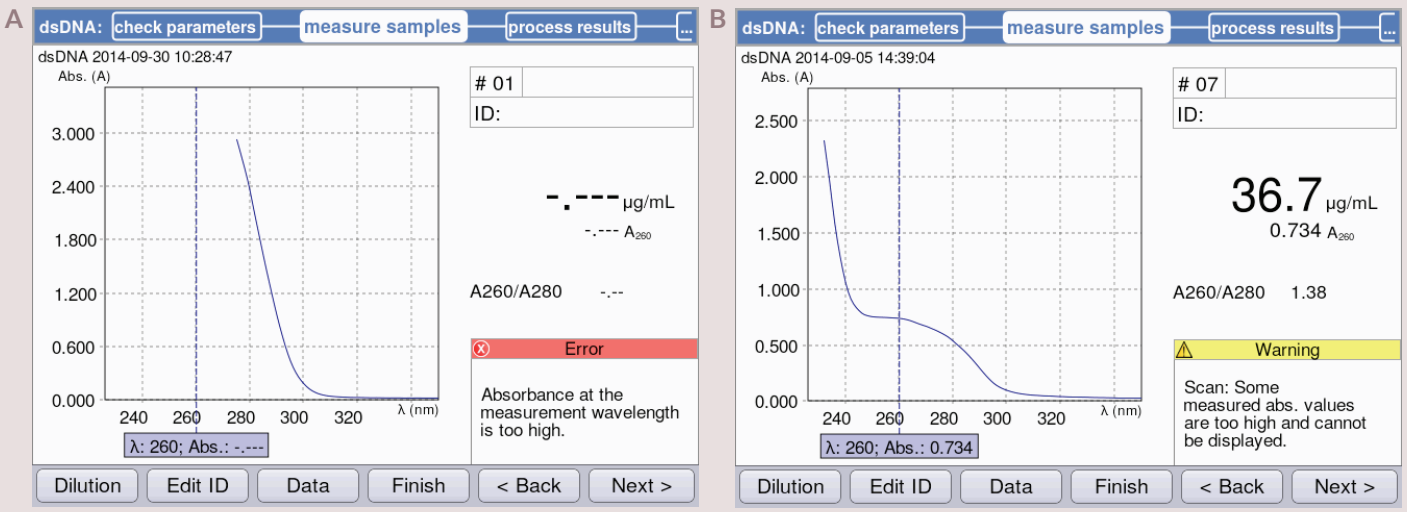


Figure 9: Error message in the results display
A: In this example, the measured value at 230 nm is too high and cannot be displayed graphically and therefore the ratio A260/A230 cannot be calculated. Nevertheless, the nucleic acid measurement at 260 nm has been carried out and the measured concentration can still be displayed. Possible reason for the abnormal shape of the spectrum (compared to a typical nucleic acids spectrum) and low A260/A280 ratio: Protein contamination.
B: In this example, the measured value of the nucleic acid sample at 260 nm is higher than 3.0 and cannot be displayed. The sample must be diluted or measured again with a cuvette with a shorter light path.

Most frequent sources of errors

The dilution	> We recommend a dilution factor of 1:10 to 1:50 at the most, as the pipetting error increases at higher dilution ratios. > Has the dilution factor been set correctly? > Check the correctness of the pipettes via calibration.
Very important! Mix the sample	> The nucleic acid sample should be briefly mixed prior to both diluting and measuring in order to avoid possible concentration gradients in the sample. > Before transferring the sample into the cuvette vortex briefly again prior to measurement to prevent concentration fluctuation caused by long storage of the sample.
The measuring medium	> The absorption behavior of nucleic acids is influenced by the pH value and the ionic strength of the buffer. One can thus only obtain precise concentrations under controlled pH conditions and using solutions with low ionic strengths, e.g. 10 mM Tris-HCl pH 8.0. Because water is not pH-stable, fluctuating measurement results may occur. > Some buffers may exhibit self-absorption in the UV range. In order to avoid inaccuracies, use the same buffer in which the sample was resuspended/eluted following isolation for the blank and the sample.
The cuvettes and what you should be aware of	> The light beam height of the cuvette must match with the one of the devices (8.5 mm for all Eppendorf photometers). > The correct layer thickness of the cuvette must be entered in the device's parameter settings. When changing from 10 mm to 2 mm or 1 mm and the other way round, the blank must be measured again. > Quartz glass cuvettes should be carefully cleaned, so that they are free of DNA/RNA contamination. > The light path of the cuvette must be oriented in the direction of the light beam of the device. > The blank and the sample should be ideally measured in the same cuvette and also in the same orientation in order to avoid fluctuations of the measured values between different cuvettes. > The specific minimum sample volume for the cuvette must be considered. For the UVette this is 50 µL. Stray light effects may occur otherwise. > Air bubbles or other visible particles in the cuvette must be avoided.
Other causes	> The device must be lying flat to ensure its correct functionality. > The cuvette shaft must be clean. > The pipettes must be clean and calibrated correctly. > The pipette piston must be clean to avoid contaminations of the sample.

Still in doubt?

Carry out a reference measurement in comparison to a standard nucleic acid sample with a defined concentration or use the UV-VIS filter set offered by Eppendorf for the various devices.

UV-VIS Filter Test

When handling errors have been eliminated, you can determine technical errors of the BioPhotometer D30 / BioSpectrometer with reference measurements in comparison with reference materials. For this purpose, Eppendorf offers Secondary UV-VIS Filter Sets. The determined measuring values are compared with the limiting values established for each filter.

This enables you to obtain information concerning the accuracy and precision of the device. You will find more information in the operating manual and tips for carrying out such a test in our userguide No. 10 [2].

Still need assistance?

Please contact your local Eppendorf organization via www.eppendorf.com.

Literature

[1] Armbrecht M, Gloe J, Goemann W: Determination of nucleic acid concentrations using fluorescent dyes in the Eppendorf BioSpectrometer® fluorescence

[2] Borrmann, L, Armbrecht. M: Evaluating the functionality of the Eppendorf Photometer® and Eppendorf BioSpectrometer® using a Secondary UV-VIS Filter Set

Ordering Information

Description	Order no. international	Order no. North America
Eppendorf BioPhotometer® D30 230 V / 50-60 Hz, mains/power plug Europe, 120 V / 50-60 Hz, mains/power plug North America	6133 000.001	6133000010
Eppendorf BioPhotometer® D30 Reference filter set	6133 928.004	6133928004
Eppendorf BioSpectrometer® basic 230 V / 50-60 Hz, mains/power plug Europe, 120 V / 50-60 Hz, mains/power plug North America	6135 000.009	6135000017
Eppendorf BioSpectrometer® kinetic, 230 V / 50-60 Hz, mains/power plug Europe, 120 V / 50-60 Hz, mains/power plug North America	6136 000.002	6136000010
Eppendorf BioSpectrometer® Reference filter set	6135 928.001	6135928001
Eppendorf BioSpectrometer® fluorescence, 230 V / 50-60 Hz, mains/power plug Europe, 120 V / 50-60 Hz, mains/power plug North America	6137 000.006	6137000014
Eppendorf BioSpectrometer® fluorescence Reference filter set	6137 928.009	6137928009
Eppendorf µCuvette G1.0 Eppendorf microvolume measuring cell for Eppendorf BioPhotometer® and Eppendorf BioSpectrometer®	6138 000.018	6138000018
UVette® 220 nm–1,600 nm Original Eppendorf disposable cuvette, individually packaged, certified PCR clean and protein-free, 80 pcs.	0030 106.300	952010051
UVette® routine pack 220 nm–1,600 nm Eppendorf Quality™ purity grade, disposable box, 200 pcs.	0030 106.318	952010069
Thermo Printer DPU-S445 incl. mains / power plug and printer cable 230 V, EU 115 V / 100 V, mains/power plug North America	6135 011.000	6135010004
Thermo paper 5 rolls	0013 021.566	952010409

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