DNA measurements in low volume samples, microplates and cuvettes on the SPECTROstar *Nano*

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Purpose

To detail how the SPECTROstar *Nano* absorbance reader measures nucleotides such as DNA in microplates, cuvettes and low volume samples with the BMG LABTECH's LVis Plate. (Fig. 1) Beer-Lambert law, purity determination, pathlength and 340 nm corrections will also be discussed.

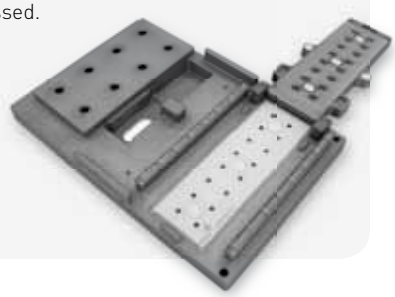

Fig. 1: BMG LABTECH's LVis Plate for low volume (2 µL) measurements and instrument performance testing

Theoretical Background

1. Absorbance measurements

Definition absorbance

Generally most substances in solution are able to absorb light at a special wavelength or wavelength range. This means that after sending a defined amount of light (I_0) through the solution, a reduced amount of light (I) will be detected afterwards.

$$\text{Absorbance (A)} = \log\left(\frac{I_0}{I}\right) \quad \textit{(equation 1)}$$

There is a direct linear relation between the absorbance and the concentration of the solute, up to certain limits. This relation is shown in the Beer-Lambert or Beer's law.

$$\text{Beer´s law: } A = b \cdot c \cdot \varepsilon \quad \textit{(equation 2)}$$

b = pathlength [cm]
c = concentration of absorbing substance in solution [mol/l or M]
ε = substance-specific constant [cm-1 M-1] (extinction coefficient)

Absorbance measurements can be performed either for the direct determination of absorbing substances, like for nucleotides and proteins, or for an absorbing dye bound to the substance of interest. In this technical application note the direct determination of DNA using absorbance is explained.

2. DNA

Present in all living organisms, DNA nucleotides consist of a sugar backbone, a base (thymine, guanine, adenine, cytosine) and a phosphate group. The nitrogen rich bases absorb light at 260 nm, this wavelength can be used to determine the DNA concentration. As DNA is so highly studied, the extinction coefficients for dsDNA, ssDNA and for RNA are widely known. The reciprocal value of the coefficient at a 1 cm pathlength can be used as a factor to determine the concentration of nucleic acids.[1]

Table 1: Reciprocal value of extinction coefficients for nucleic acids

Nucleic acids	1 / Extinction coefficient [µg/mL][1]
double stranded DNA	50
single stranded DNA	33
RNA	40

The extinction coefficients enable DNA measurements without preparing a standard curve.

[1] Using a 1-cm pathlength of light, the extinction coefficient for nucleotides at 260 nm is 20 per cm per M. Based on this the absorbance at 260 nm in a 1-cm quartz cuvette of a 50 µg/mL solution of double stranded DNA, 33 µg/mL solution of single stranded DNA or a 40 µg/mL solution of single stranded RNA are all equal to 1 OD.

3. Pathlength Correction

With the SPECTROstar *Nano* it is possible to measure in cuvettes and also in microplates. Cuvette-based measurements show the advantage that all absorbance values are automatically normalized to 1 cm. These values can be directly used to calculate the DNA concentration using Beer's law (equation 2) and known extinction coefficients (Table 1).

In microplates the pathlength will vary, depending on the volume of liquid in the well along with the height and dimensions of the well (i.e. 96- versus 384-well plates). To obtain data that can be used in Beer's law it is necessary to normalize the absorbance results to a 1 cm pathlength (b in equation 2). Pathlength correction can be achieved through a number of methods:

1. **Use a microplate with a defined pathlength (e.g. LVis Plate, pathlength = 0.5 mm).** OD measurements are taken and a standard multiplication value is then applied to achieve a 1cm pathlength.
2. **Use of the pathlength correction feature in the software for standard microplates.** The volume and microplate used is specified in the test protocol and an algorithm will then be applied to the data.
3. **Use a known water peak value correction to normalize the data.** The water pathlength correction model uses a known OD value for water at a 1cm pathlength.

(equation 3)

$$\text{Pathlength of Sample} = \frac{\text{(A977-A900) Sample}}{\text{(A977-A900) 1cm (Water/Buffer*)}}$$

* = Correction factor

One advantage of using a water peak correction over other methods is that a pathlength can be created for each well allowing different volumes to be dispensed into one microplate if needed, more importantly this method will also correct for pipetting errors that may occur during sample preparation.

4. Purity Determination

DNA samples can contain impurities that will affect the values at 260 nm. Therefore it is recommended to also measure wavelengths where impurities have an absorbance maximum:

1. Contamination by protein (280 nm)
2. Contamination by phenolate, thiocyanate (230 nm)
3. Scattering of light caused by particulates (340 nm)

A common purity check is to calculate the 260/280 ratio in order to look for protein contaminations. Pure DNA gives a ratio between 1.8 – 2.0, whereas pure RNA shows ~ 2.0. High quality samples should be above 1.7. Another commonly used step is to correct OD values for scattered light by subtracting the OD value at 340 nm from the OD values at 260 or 280 nm, this greatly reduces the standard deviation and increases the sensitivity of measurements.

Materials and Methods

- UV-Cuvettes half semi-micro from Brand #759150
- UV Star 96-well plate from Greiner #655801
- Black 384-well LoBase plate µClear, COC with UV bottom from Greiner #788876
- SPECTROstar *Nano* absorbance reader equipped with LVis Plate (Fig. 2)

Herring Sperm dsDNA and HPLC grade water was obtained through normal distribution channels.

Fig. 2: BMG LABTECH's SPECTROstar *Nano* absorbance reader

Results

1. DNA measurements on the SPECTROstar *Nano* using the LVis Plate

The LVis Plate is a special microplate that contains 16 sites suitable for measuring 2 µL samples. After pipetting onto the microdrop well on the LVis Plate, the lid is closed resulting in a pathlength of 0.5 mm being generated for all 16 sites. A dsDNA standard curve for measurements with the LVis Plate is shown in Fig. 3.

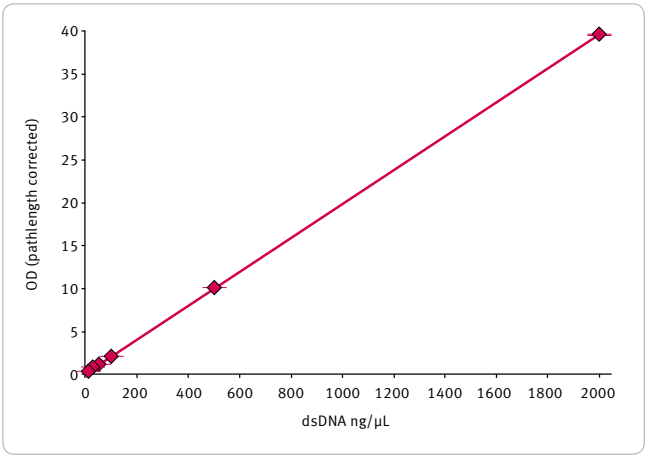

Fig. 3: DNA standard curve obtained using the LVis Plate.

Sensitivity values (LOD) will depend on how reliably the blank can be measured. The DNA sensitivity for the LVis Plate was calculated to be < 2 µg/mL.

In addition to highly sensitive measurements, the SPECTROstar *Nano* takes data points extremely fast. In only 13 seconds a full UV/Vis absorbance spectrum for all 16 sites in the LVIs plate is obtained with a resolution of 1 nm!

Table 2 shows how long LVis plate measurements take when measuring a full spectrum scan between 220-1000 nm. In addition to that read times measuring the entire 96-well and 384-well plates are presented.

Table 2: SPECTROstar *Nano* read times

SPECTROstar *Nano*	LVis Plate (16 samples)	96-well plate	384-well plate
Full UV/Vis absorbance spectrum 220-1000 nm, 1 nm resolution	13 seconds	38 seconds	250 seconds

These very fast read times enable kinetic measurements while taking a full UV/VIS absorbance spectrum and making it possible to follow a spectral shift over time. For unknown samples there is no need to select or guess the optimal wavelengths because they are all given in less than one second per well.

2. DNA measurements on the SPECTROstar *Nano* using a standard microplate

In microplates the best sensitivity is achieved when the highest possible volume is used generating a longer pathlength. This is OK generally but if sample is limited then users will usually want to use as low of a volume as possible. Therefore, it is necessary to find the best compromise between volume and sensitivity. Table 3 shows the limit of detection obtained for different volumes in different microplates.

For the measurements, a standard 96-well UV microplate from Greiner was used as well as a black LoBase 384-well microplate, a plate with UV transparent bottom. Table 3 shows that correcting the data at 340 nm generally leads to a higher sensitivity.

Table 3: dsDNA sensitivity in 96-well and 384-well plates

Plate Format	Volume	LOD in µg/mL based on A_{260}	LOD in µg/mL based on A_{260}-A_{340}
96	350 µL	0.39	0.24
96	300 µL	0.22	0.20
96	200 µL	0.45	0.36
96	100 µL	1.03	0.82
96	50 µL	1.3	1.3
384	20 µL	1.9	0.53
384	5 µL	2.8	2.4
384	3 µL	2.7	2.4

3. DNA measurements on the SPECTROstar *Nano* using a cuvette

Measuring the DNA content in cuvettes is still common in labs if only a limited number of samples need to be determined. Cuvette measurements have the advantage that the resulting absorbance values are already normalized to 1 cm. Table 4 shows results from dsDNA measurements in cuvettes.

Table 4: dsDNA results in cuvettes

dsDNA Concentration µg/mL	OD Blank-corrected at 260 nm	OD Blank-corrected A_{260}-A_{340}
100	2.015	2.002
50	1.028	1.021
25	0.523	0.516
12.5	0.266	0.262
6.25	0.136	0.134
2.5	0.053	0.053
1.25	0.030	0.028
0.5	0.012	0.012
0.25	0.007	0.006
0.05	0.004	0.003

A semi-micro UV cuvette was used to perform the measurements. A disadvantage is that at least a volume of 750 µl of dsDNA sample is necessary for each and every cuvette test. However, a high sensitivity can be obtained using cuvettes as presented in Fig. 4.

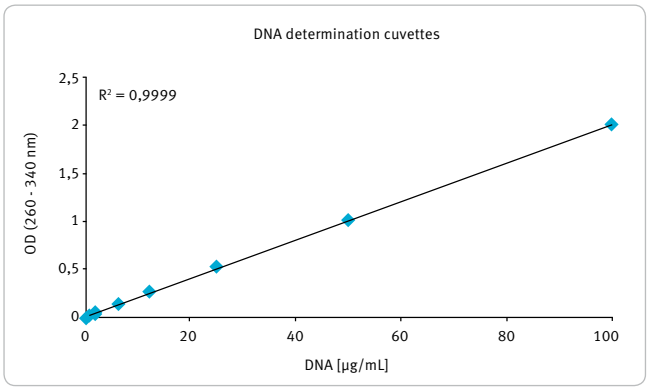

Fig. 4: DNA standard curve obtained in cuvettes.

As noted in Table 4 and Figure 4, a high linearity (R^2 = 0.9999) in the low DNA concentration range is achieved in cuvettes. A blank-correction and A_{260} - A_{340} referencing is recommended to obtain the highest sensitivity.

Conclusion

There are different possibilities to measure DNA samples using the SPECTROstar *Nano*. Table 5 shows the linear range for DNA measurements in cuvettes, standard microplates and in the LVis Plate.

Table 5: Sensitivity data and linear range for DNA measurements using the SPECTROstar *Nano*.

	LVis Plate	96-well	96-well	384-well	Cuvette
Sample volume	**2 µL**	**350 µL**	**50 µL**	**20 µL**	**800 µL**
Linear range	2 – 2500	0.5 – 100	1.5 – 1000	2 – 200	0.2 – 150
LOD in µg/mL	<2	0.24	1.3	0.55	0.2

If there is enough DNA material available and only a few samples that should be measured, cuvette measurements can be performed. For higher throughput a microplate should be used. If sample volume is very limited, then the LVis Plate is recommended as it offers a great linear range, a high sensitivity and only 2 µL of sample is needed.

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