

- The NanoPhotometer® -

A synonym for spectrophotometric quantification of submicroliter and standard sample volumes with high reproducibility and linearity over a broad range

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Introduction

The spectrophotometrical analysis of nucleic acids, proteins and bacterial cell cultures is part of the daily routine in every modern laboratory. The universal NanoPhotometer® from Implen combines measurements of submicroliter sample volumes (starting with 0.7 µl) and standard sample volumes. The submicroliter measurements are performed cuvetteless with the integrated LabelGuard™ Microliter Cell (Figure 1 and Figure 2). Higher volumes are measured with commercially available cuvettes.



Figure 1: NanoPhotometer® with integrated LabelGuard™ Microliter Cell. Insert: Pipetting of a sample on the measurement window.

Due to its approach the NanoPhotometer® is an all-rounder for the various spectrophotometrical applications in a modern laboratory. Based on the available methods for single/multiple wavelength and concentration measurements, full spectrum scans, standard curve determinations as well as ratio calculations and even kinetics, the user can create all kinds of customized applications for their individual needs. Predefined methods and functions are available for nucleic acid analysis (dsDNA, ssDNA, RNA, oligos), determination of the labeling efficiency (dye incorporation rate e.g. for microarray applications), protein quantification (Bradford, Lowry, BCA, Biuret, Protein A280 (according to Christian-Warburg)) and cell density measurements (OD 600).

In addition to a built-in printer option the NanoPhotometer® can be linked to a computer network via USB or Bluetooth connection for data export and printing options. Due to its versatility and the possibility to measure submicroliter sample volumes the NanoPhotometer® is a very useful analysis tool for technologies like Real-Time PCR, Laser Capture Microdissection and Microarray based applications.

Technical specifications

The available spectral scan range of the NanoPhotometer® is 200 nm to 950 nm, the wavelength range for single and multiple wavelength measurements is between 190 nm and 1,100 nm. The average dynamic range for undiluted dsDNA is between 15 ng/µl and 4,000 ng/µl (optional extensions for 7 ng/µl to 8,000 ng/µl are available), for proteins it is between 0.2 mg/ml and 100 mg/ml (e.g. BSA solution, also extendable).

Table 1: Specifications

	lid 5	lid 10	lid 50	lid 100
pathlength [mm]	2	1	0.2	0.1
virtual dilution	1:5	1:10	1:50	1:100
sample volume [µl]	6 - 10	3 - 5	0.7 - 4	0.7 - 3
dynamic range dsDNA [ng/µl]	7 - 350	15 - 700	250 - 4,000	500 - 8,000
sample recovery	yes (no evaporation)			
cleaning time	5 sec			
sample through-put	160/h			
compatible with volatile solvent (e.g. acetonitrile /toluene)	yes			

Experimental specifications

High reproducibility and linearity over a broad concentration range are basic requirements for a reliable spectrophotometrical sample analysis. In this application note we describe the performance of the NanoPhotometer® in terms of reproducibility and linearity for nucleic acid and protein samples as well as for cell density measurements of bacterial cultures.

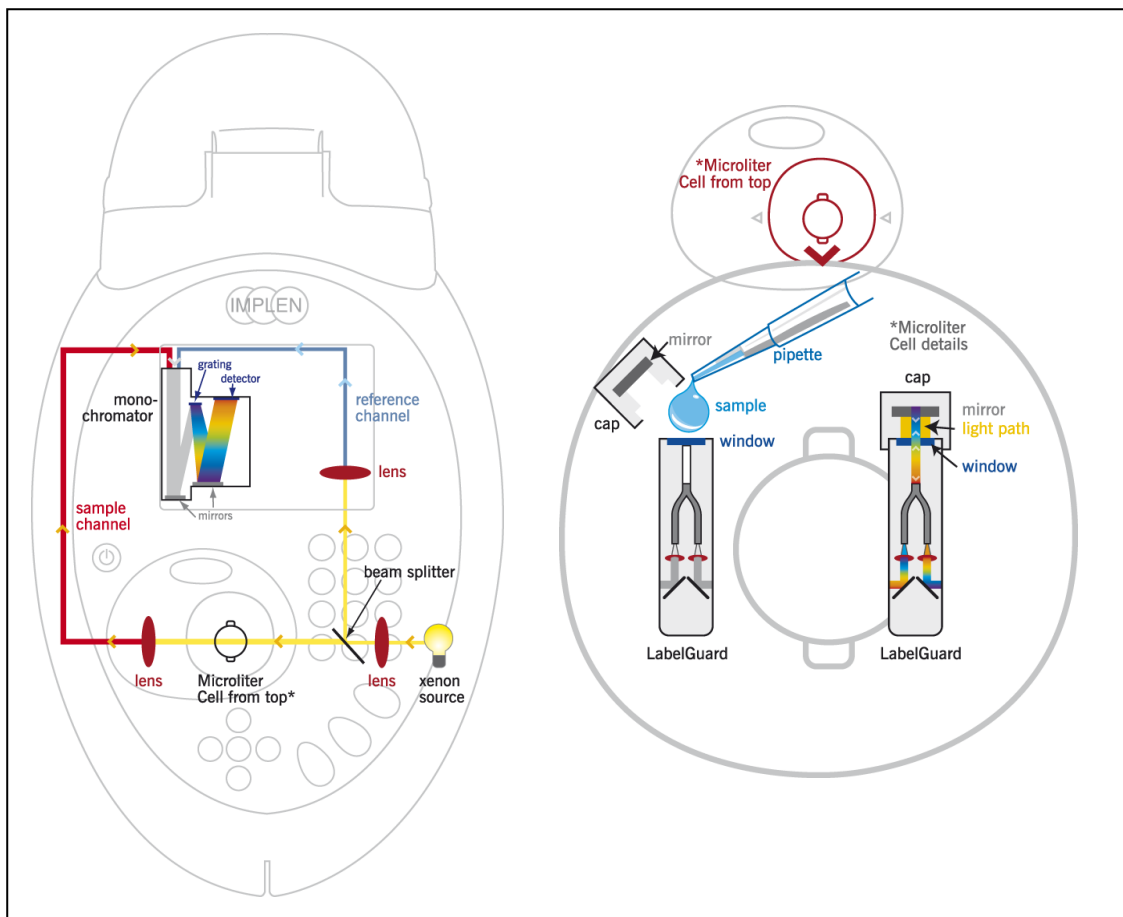


Figure 2: Optical path of the NanoPhotometer®

Due to the integrated beam deflexion and the use of fiber-optic light conductors the sample can be measured directly on the measurement window. Using one of the four available lids of the microliter cell (lid 10, lid 50 and optional lid 5 or lid 100) different liquid columns of defined pathlength are created (Table 1). Virtual dilutions of up to 1/100 are generated in comparison to a standard 1 cm cuvette measurement, without a manual dilution of the sample. Dilution errors are avoided and the sample can be retrieved after the measurement.

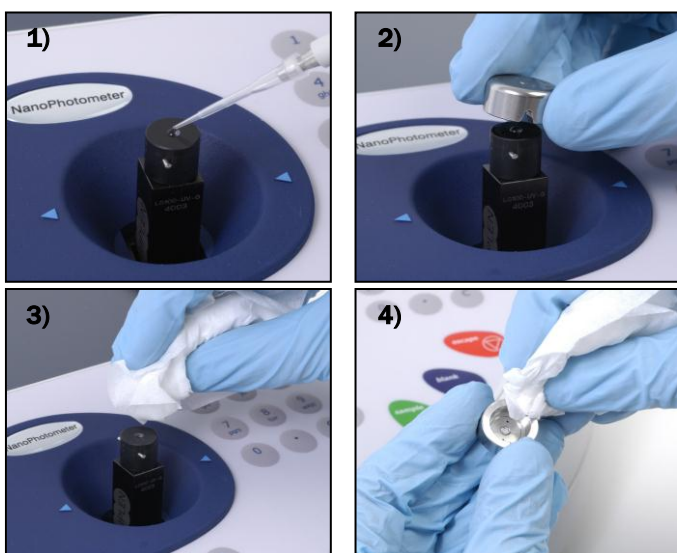


Figure 3: Cuvetteless Measurement

1) Pipetting of the sample onto the centre of the measuring window. 2) Closing of the lid. 3) Cleaning of the measuring window with a fluff free tip or Kimwipe. 4) Removing of sample residues from the lid mirror with a fluff free tip or Kimwipe.

The linearity for different samples was verified utilizing an ATP solution, plasmid DNA, genomic DNA, an *Escherichia coli* culture and an immunoglobulin G (IgG) solution representing proteins. This specific setup of samples has been chosen to demonstrate the multiple applications which can be covered with the NanoPhotometer®. Every sample was serially diluted and independent multiple measurements of the optical density were carried out. After each measurement, the measurement window was cleaned with a dry Kimwipe (Figure 3) or the cuvette was changed and a new aliquot of the sample was pipetted. For nucleic acid and protein quantification the "Background Correction-Function" was turned on. To quantify ATP solution (100 mM), seven different dilution steps were generated. Ten independent measurements were taken from each dilution with lid 50 at 260 nm. The sample volume was set to 2 µl. The resulting data shows a very good linearity with a coefficient of determination of $R^2=0.9997$ (Figure 4A). According to the Lambert-Beer law ($\epsilon=E/c*d$) an ATP extinction coefficient of $15.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ can be calculated based on this data. This value corresponds with the known ATP extinction coefficient of $15.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ determined in 1965 by Bock et al..

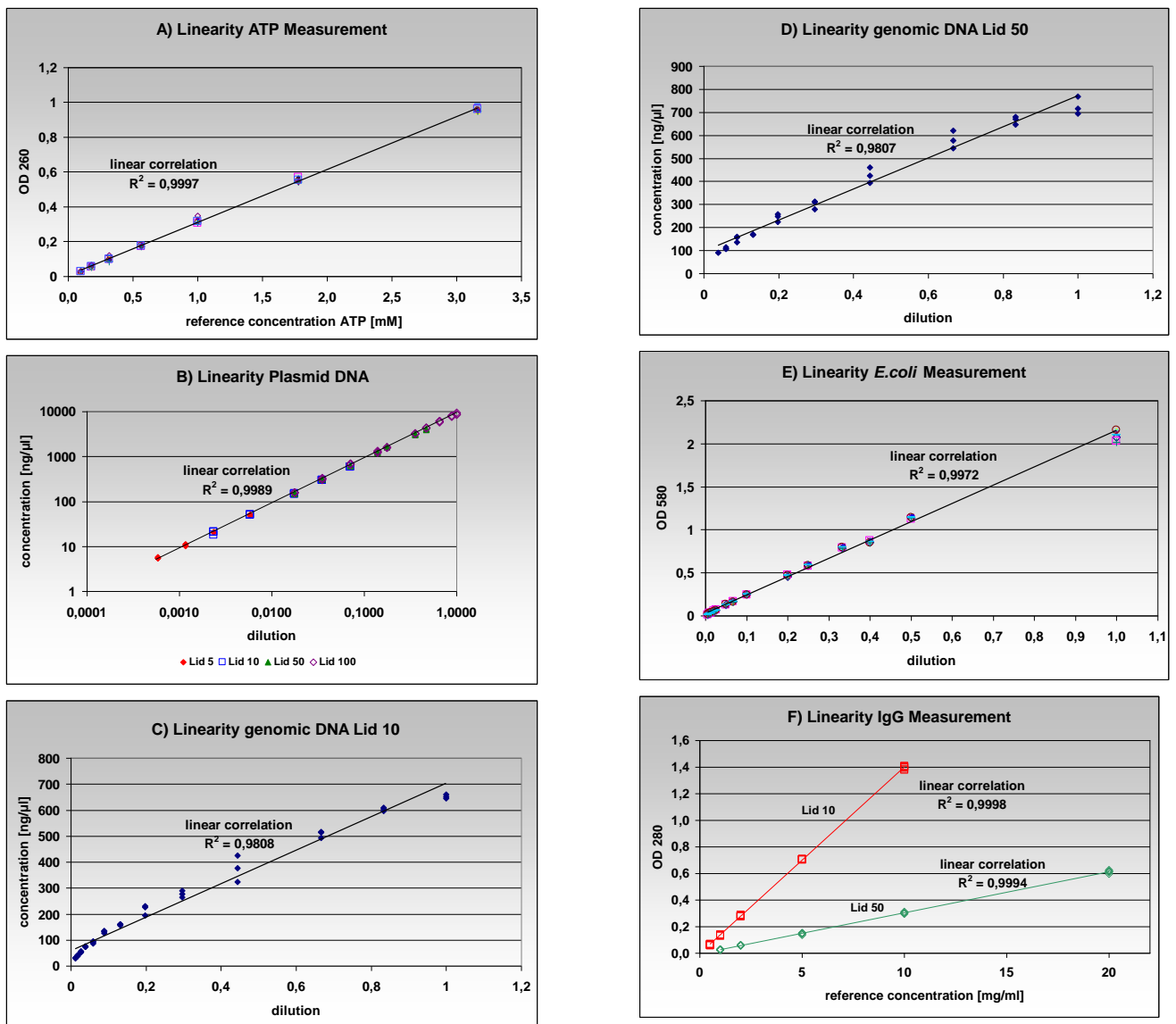


Figure 4: Linearity

An ATP solution, plasmid DNA, genomic DNA, *E. coli* culture and immunoglobulin G (IgG) solution were quantified to determine the linear range of the NanoPhotometer®. **A)** Seven dilution steps of an ATP solution were measured cuvetteless (lid 50) at 260 nm. Each dilution was measured 10 times. **B)** Triplicates of the plasmid DNA were measured at 14 dilution steps at 260 nm. The measurements were carried out cuvetteless. According to their dynamic range the corresponding lids were chosen. **C) and D)** The concentration of the genomic DNA was determined in 13 dilutions with lid 10 and lid 50 at 260 nm. Each dilution was measured in triplicates. **E)** The *E. coli* culture was measured in 14 dilution steps, each 8 times at 580 nm with 1 ml semi-micro plastic cuvettes. **F)** The concentration of the IgG solution was determined cuvetteless in 5 dilution steps at 280 nm (lid 10 and lid 50). Five independent measurements were performed with each dilution.

Plasmid DNA (5,100 bp) was measured over a very broad concentration range starting from 5 ng/μl up to 8,500 ng/μl at 260 nm. 14 dilutions were quantified and each dilution was measured in triplicates with the corresponding lid of the Microliter Cell (Table 1). An overlapping dynamic range was measured with the equivalent lids. The calculated coefficients of determination (R^2) for all lids were approximately 1 (Table 2). In addition, it could be proved that the measured concentrations, where overlapping, were independent of the lid. Moreover, even over this challenging concentration range, a linear correlation exists (Figure 4B).

To quantify genomic DNA, 13 dilutions were measured in triplicates with lid 10 and lid 50 at 260 nm.

The concentration range was selected between 30 ng/μl

Table 2: Coefficients of determination of plasmid DNA

Lid 5	Lid 10	Lid 50	Lid 100
$R^2 = 1$	$R^2 = 0,999$	$R^2 = 0,998$	$R^2 = 0,998$

and 700 ng/μl. The sample volume was 3 μl for both lids. Genomic DNA is difficult to measure because of the higher viscosity of the sample and the tendency to stick to surfaces. This validates the slightly lower coefficients of determination observed with these samples using both lids ($R^2=0.9807$). Despite these obstacles, we were able to demonstrate a very good

linear correlation with this sample (Figure 4C and 4D).

The linearity measurements for bacterial cultures were performed with an *E. coli* culture. 14 dilutions were set up and each dilution was measured eight times at 580 nm with semi-micro plastic cuvettes (volume 1 ml). The resulting coefficient of determination (R^2) was 0.9972 and confirms the high linearity of the photometer (Figure 4E). Similar experiments performed with *Rhodobacter capsulatus* were also consistent with these results (data available upon request from the authors).

To demonstrate the linearity with a protein sample, an IgG dilution series was selected. Five dilutions were set up and each dilution was measured cuvetteless 5 times at 280 nm (lid 10, lid 50). The sample volume was 3.5 μ l. The excellent coefficients of determination (R^2) were 0.9998 and 0.9994 (Figure 4F). Besides, in a separate experimental set up it was also shown that the NanoPhotometer[®] is capable for higher protein concentrations because the function of the measurement window is independent of surface tension (data available upon request from the authors).

The excellent reproducibility of the NanoPhotometer[®] could be demonstrated with repeated measurements of plasmid DNA, genomic DNA, *E. coli* suspension and IgG solution. The coefficients of variation (c.v.) for the plasmid DNA ranged at a very low level between 0.5 % and 2.8 % (Table 3). However, the coefficients of variation values for genomic DNA varied at a higher range between 3.6 % and 4.0 % using lids 10 and 50 respectively (Table 4). Considering the fact that the measurement of genomic DNA is difficult due to the nature of the samples, the observed coefficients of variation are in an excellent range. Besides, these values were obtained without any prior heating or pretreatment of samples.

The 14 dilution steps of the *E. coli* culture were measured 8 times each. The standard deviations of the 8 independent measurements are minimal and emphasize the high reproducibility of the photometer (Figure 5A, error bars).

Analyzing the IgG solution confirms a minimal standard deviation of the single measurements from the mean for all measurements (Figure 5B, error bars). In addition the difference between the reference concentration and the measured concentration is also very small.

Table 3: Reproducibility of plasmid DNA

No.	low concentration [ng/ μ l]	medium concentration [ng/ μ l]	high concentration [ng/ μ l]
1	50	294	1,620
2	52	294	1,723
3	50	295	1,635
4	49	294	1,592
5	50	291	1,630
6	51	295	1,598
7	51	292	1,580
8	52	294	1,575

9	52	292	1,562
10	51	291	1,598
Mean	51	293	1,611
StDev	1.0	1.5	45.8
c.v. %	1.9	0.5	2.8

Table 4: Reproducibility of genomic DNA

No.	lid 10 concentration [ng/ μ l]	lid 50 concentration [ng/ μ l]
1	593	682
2	583	642
3	574	638
4	578	648
5	606	668
6	619	638
7	622	708
8	629	690
Mean	601	664
StDev	21.4	26.8
c.v. %	3.6	4.0

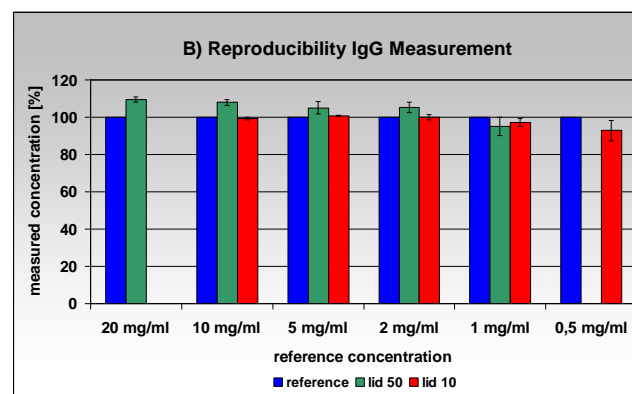
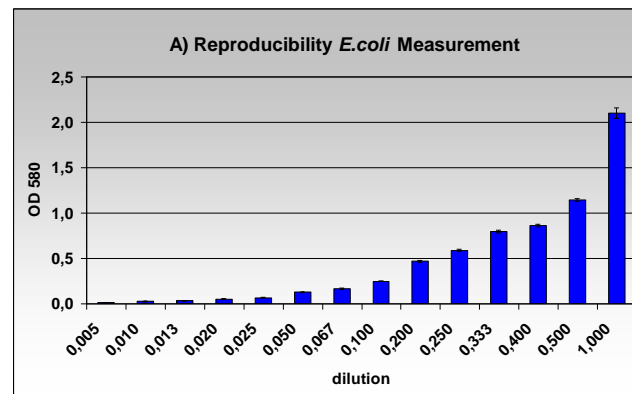


Figure 5: Reproducibility

The reproducibility of the NanoPhotometer[®] as demonstrated for an *E. coli* culture and an IgG solution. **A)** Fourteen dilution steps of the *E. coli* culture were measured at 580 nm with semi-micro plastic cuvettes. Each dilution was measured 8 times. With optical densities between 0.015 and 2.1, the quantification was carried out over the whole absorbance range. The recorded mean values of the 8 measurements are shown (blue columns). The standard deviation of the 8 measurements is indicated by the error bars. **B)** Six dilutions of the IgG solution were measured cuvetteless with lid 10 and lid 50 at 280 nm. Five independent measurements have been carried out for each dilution. The reference values are set as 100 % (blue columns) vs. the measured concentrations for lid 50 (green) and lid 10 (red). The error bars indicate the standard deviation for the 5 independent measurements vs. the mean average.

An additional important application built into the NanoPhotometer® is the quantitative and qualitative analysis of fluorescent dye labelled samples. In addition to the common cyanine dyes (Cy3, Cy3.5, Cy5 and Cy5.5), different AlexaFluorophore, Oyster-Dyes and Texas Red can be measured.

To analyze the performance for the determination of the incorporation rate for Cy3 and Cy5, 10 aliquots of genomic DNA were labelled using the Agilent labelling protocol for Array CGH. The samples were measured at the corresponding wavelengths (Figure 6). Table 5 shows the quantity and quality of the DNA used for the experiment as well as the dye concentration and incorporation rate.

During the experiment the time per measurement for the NanoPhotometer® was also monitored. The average total handling time per sample was determined to be 20 seconds including a measurement time of less than 4 seconds.

Table 5: Incorporation of Cy3 and Cy5 dyes in genomic DNA

dsDNA [ng/ul]	dsDNA [µg]	Cy3 [pmol/ul]	Cy5 [pmol/ul]	Cy dye Incorporation rate [pmol/ug]
107	5,350	-	2.2	20.9
105	5,250	-	2.6	24.8
92	4,600	-	1.8	19.6
113	5,650	-	2.9	25.7
105	5,250	-	2.7	25.7
110	5,500	-	2.9	26.4
107	5,350	-	2.5	23.4
98	4,900	-	2.7	27.6
118	5,900	-	2.7	22.9
111	5,550	-	2.0	18.0
110	5,500	3.2	-	29.1
99	4,950	2.4	-	24.2
102	5,100	3.2	-	31.4
88	4,400	1.9	-	21.6
108	5,400	3.3	-	30.6
108	5,400	3.2	-	29.6
120	6,000	3.7	-	30.8
112	5,600	3.0	-	26.8
114	5,700	3.4	-	29.8
110	5,500	3.2	-	29.1

Conclusion

The NanoPhotometer® from Implen counts for high accuracy, robustness and absolutely reliable and precise results for submicroliter and cuvette measurements. Simple sample handling for very low and standard volumes (0.7 to 3,500 µl) as well as high throughput rates due to very short measuring times are the major advantages of the system. The NanoPhotometer® offers an unmatched dynamic range and a very high reproducibility of measurements. With its maintenance free setup, the NanoPhotometer® helps to reduce cost and down times.

The NanoPhotometer® will serve as a powerful tool in every genomic or proteomic laboratory engaged in nucleic acid and protein based research, microarray

analysis as well as for the analysis of bacterial cell cultures.

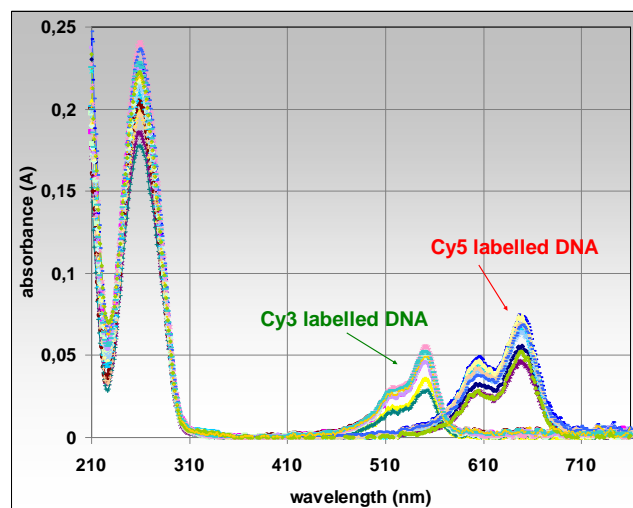


Figure 6: Spectrum of labelled genomic DNA

Samples of genomic DNA labelled with cyanine 3 (green) or cyanine 5 (red) were measured over a wavelength range from 210 nm to 760 nm with the NanoPhotometer®.

Literature

Bock et al., (1956), Arch. Biochem. Biophys. 62: 253-264

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