Applications

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PCR and real-time PCR experiments performed with DNA samples which have undergone multiple measurements in the UVette®, using the Eppendorf BioPhotometer plus™

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Abstract

DNA samples were measured multiple times in three different concentrations using the BioPhotometer plus and subsequently employed directly in real-time PCR and standard PCR experiments. These experiments did not detect any loss of quality of the DNA due to UV light as compared to unmeasured controls. Based on these results, we conclude that DNA samples which have been measured in the Eppendorf BioPhotometer may be used in suitable downstream applications.

Introduction

When the need for photometric determination of DNA concentrations arises, it is possible that samples are available only in low concentrations or small amounts. Frequently, the sample volume is so small (e.g. 50 µL) that there is only sufficient sample material for one measurement in a small volume cuvette such as the Eppendorf UVette. Microliter measuring systems such as the Hellma TrayCell or the Nanodrop by Thermo Scientific cannot be used in this case, as their light paths are very short and therefore not suitable for the measurement of low concentration DNA samples.

Which options are available when the reproducible determination of nucleic acid concentrations is crucial for subsequent applications?

In order to answer this question, we are going to demonstrate that it is possible to use a DNA sample whose concentration has been determined in a 10 mm light path for subsequent applications, using real-time PCR and standard PCR experiments. The goal is the evaluation whether DNA samples which have been measured multiple times with UV light yield the same results as unmeasured samples. DNA strand breaks brought about by UV light would lead to shorter PCR products, which would be visible with the detection methods used.

For the photometric determinations, Eppendorf UVettes with an optical light path of 10 mm were used.

Materials and Methods

Multiple measurements of DNA samples with different concentrations, and subsequent use in real-time PCR and standard PCR

Materials

Eppendorf BioPhotometer plus
Eppendorf UVette
Eppendorf Mastercycler pro
Eppendorf Mastercycler ep realplex
Eppendorf twin.tec PCR Plates
Eppendorf ep*Motion* 5075 LH
Eppendorf Centrifuge 5804
Heat Sealer
Heat Sealing Film
Eppendorf Research Pipette
Eppendorf epT.I.P.S.
Humane genomische DNA (Roche, approx. 200 ng/µL)
λ-Bakteriophagen-DNA (Roche, approx. 200 ng/µL),
Invitrogen eGel 1 % (G7208-02)
UVP-BioDoc-IT-System

Setup real-time PCR mit λ-DNA	
	Final concentration (per 20 µL reaction)
Primer 1: Lambda F2 5'-cgcacaggaactgaagaatg-3'	300 nM
Primer 2: Lambda R2 5'-ccgtcgagaatactggcaat-3'	300 nM
QuantiFast SYBR Green PCR Kit (Qiagen)	1x
λ-Bakteriophagen-DNA	0,00025 ng/µL
H_2O	ad 20 µL

Setup standard-PCR (beta-Globin)	
	Final concentration (per 20 µL reaction)
Primer 1: forward 5'-ggttggccaatctactcccagg-3'	500 nM
Primer 2: reverse 5'-gctcactcagtgtggcaaag-3'	500 nM
5X Green GoTaq reaction Buffer	1x
$MgCl_2$	2,5 mM
dNTP's	200 µM
GoTaq Hot Start Polymerase (Promega)	0,025 U/µL
Human Genomische DNA	0,25 ng/µL
H_2O	ad 20 µL

Methods

3 serial dilution steps of approximately 50, 25 and 10 ng/µL DNA were generated from samples of λ- bacteriophage DNA and human genomic DNA, respectively. Each of these 3 dilutions were transferred to the UVette and measured 10 consecutive times in the BioPhotometer plus using the method dsDNA. 60 µL of the sample were removed prior to the first, after the 5th and after the 10th measurement.
For real-time PCR, all samples were diluted to a concentration of 0.001 ng/µL, using the ep*Motion* 5075 LH. Subsequently, 15 µL of the PCR reaction mix described above were transferred into an Eppendorf twin.tec Plate, followed by addition of 5 µL of the diluted DNA solution. Thus, approximately 0.005 ng λ-DNA (approx. 7.5 * 10^5 copies) were used per reaction. 3 x 5 µL of each sample were removed for the PCR, so that altogether 27 samples were run. As a negative control, an additional 3 PCR reactions were supplied with 5 µL water each.
The real-time PCR was performed in the Eppendorf Mastercycler ep realplex using the following program:

95 °C - 5 min
95 °C - 10 s
60 °C - 30 s } x40
95 °C - 15 s
60 °C - 15 s
10 min to 95 °C
95 °C – 15 s

For standard PCR, all samples were diluted to approximately 1 ng/µL using an ep*Motion* 5075 LH. Subsequently, 15 µL of the PCR reaction described above were transferred to an Eppendorf twin.tec Plate and supplemented with 5 µL of the diluted DNA solution, yielding a final concentration of approximately 5 ng DNA per reaction. 3 x 5 µL of each sample were used for the PCR, leading to a total of 27 reactions. Identical to the real-time PCR, 3 reactions supplemented with 5 µL water each were used as negative controls.
The following PCR was performed in an Eppendorf Mastercycler pro using the program described below:

95 °C - 2 min
95 °C - 15 s
56 °C - 15 s } x30
72 °C - 30 s
72 °C - 5 min
10 °C – ∞

Following the PCR, all samples were transferred to an Invitrogen eGel, and the image was subsequently analyzed on a gel documentation platform (UVP-BioDoc-IT-System).

Results and Discussion

Multiple measurements of DNA samples of different concentrations, and subsequent use in real-time PCR and standard PCR

The aim of this experiment was to verify whether it was generally possible to measure the DNA concentration of a sample photometrically in the UVette, using the BioPhotometer plus, and to use this same sample directly in subsequent applications such as PCR. In the past, we were able to demonstrate the feasibility with microarray experiments, where the entire sample could be used for further microarray processing following concentration determination in the UVette [2].
Direct re-use of the sample is especially important in cases where only low-concentration DNA samples, for example in the range of 5 ng/µL, are available, which need to be determined as accurately as possible. The attempt to analyze these samples with the help of alternative systems which utilize shorter path lengths, carries the risk of inaccurate measurements, as these samples are clearly below the recommended concentration range. For example, when using a 2 mm light path, 5 ng/µL dsDNA correspond to an absorption value of 0.02; when using a light path of 1 mm, the absorption value is 0.01, and a 0.2 mm light path will just yield an absorption of 0.002. According to the literature, absorption of DNA solutions should always be above 0.1 [1] in order to be able to ensure reproducibility. However, even for higher concentrated DNA solutions, such as plasmid preparations, the time savings are considerable, as no additional dilution steps are necessary when using the sample directly.

With regards to sample recovery, samples may be easily aspirated from the UVette using a pipette, aided by the relatively large sample volume of 50 µL. In comparison, microliter measuring systems utilize much smaller volumes, such as 2 µL, thus rendering sample recovery much more difficult. Furthermore, use of the UVette prevents contamination with preceding samples when the sample is to be recovered.

As mentioned in the methods, unmeasured DNA samples were compared to DNA samples of three different concentrations, which had been measured 5 and 10 times photometrically, using real-time and standard PCR.

In order to be able to compare the results, all samples deemed for PCR were calibrated to a uniform concentration using the Eppendorf ep*Motion* 5075 following the photometric measurements. A total of 30 samples, consisting of 3 replicates each per concentration and number of measurements, plus 3 negative controls, were prepared for the PCR runs.

The use of the automated pipetting system ep*Motion* served the purpose of eliminating possible individual pipetting errors which may have an impact on the results of the real-time PCR.

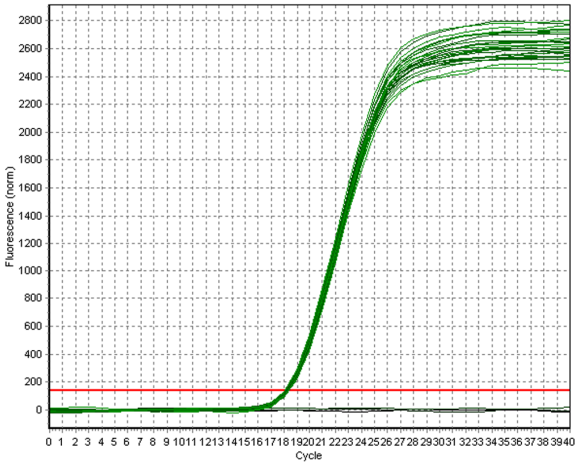

Figure 1: Result of the real-time PCR using Lambda-DNA
The PCR products were detected with SYBR-Green.

Figure 1 shows the results for the real-time PCR experiments. The amplification curves of all 30 PCR runs are displayed in parallel.

Table 1: C_t-values of the individual PCR runs using Lambda-DNA

Position in the plate	Sample name	Number of measurements in the BioPhotometer plus	C_t-value SYBR	average C_t- value SYBR	Standard deviation C_t-value
A1	Negative control	0	-		
A2	Negative control	0	-		
A3	Negative control	0	-		
A4	High DNA-concentration	0	18.30	18.27	0.02
A5	High DNA-concentration	0	18.26	18.27	0.02
A6	High DNA-concentration	0	18.26	18.27	0.02
A7	High DNA-concentration	5	18.35	18.20	0.15
A8	High DNA-concentration	5	18.04	18.20	0.15
A9	High DNA-concentration	5	18.22	18.20	0.15
A10	High DNA-concentration	10	18.28	18.29	0.10
A11	High DNA-concentration	10	18.20	18.29	0.10
A12	High DNA-concentration	10	18.39	18.29	0.10
B4	Medium DNA-concentration	0	18.05	18.10	0.06
B5	Medium DNA-concentration	0	18.17	18.10	0.06
B6	Medium DNA-concentration	0	18.09	18.10	0.06
B7	Medium DNA-concentration	5	18.09	18.23	0.15
B8	Medium DNA-concentration	5	18.39	18.23	0.15
B9	Medium DNA-concentration	5	18.20	18.23	0.15
B10	Medium DNA-concentration	10	18.25	18.24	0.15
B11	Medium DNA-concentration	10	18.09	18.24	0.15
B12	Medium DNA-concentration	10	18.39	18.24	0.15
C4	Low DNA-concentration	0	18.13	18.07	0.05
C5	Low DNA-concentration	0	18.05	18.07	0.05
C6	Low DNA-concentration	0	18.03	18.07	0.05
C7	Low DNA-concentration	5	18.25	18.23	0.09
C8	Low DNA-concentration	5	18.12	18.23	0.09
C9	Low DNA-concentration	5	18.30	18.23	0.09
C10	Low DNA-concentration	10	18.29	18.38	0.10
C11	Low DNA-concentration	10	18.49	18.38	0.10
C12	Low DNA-concentration	10	18.37	18.38	0.10

Since almost identical C_t values with minimal deviations were obtained for the reactions (table 1), it can be concluded that the respective DNA samples were recovered unharmed after the measurements in the UVette with the BioPhotometer plus, independent of concentration. The melting curve analysis, shown in figure 2, shows a comparable pattern between the unmeasured and the measured samples. Shorter DNA fragments, which could be expected due to template strand breaks, would have been evident based on lower melting points.

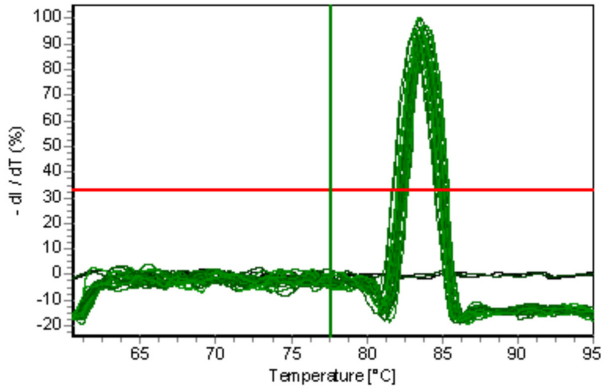

Figure 2. Result of the melting curve analysis
The shape of the curve is homegeneous, which indicates equal length of DNA products.

In addition to the real-time PCR experiments, a standard PCR with a longer PCR target was subsequently performed. Following the electrophoretic separation of the PCR products in an agarose gel (fig. 3), comparable results were obtained for the unmeasured samples and the photometrically measured samples with regards to quality and quantity.
No degradation of DNA could be detected as a result of UV measurements in the BioPhotometer plus.

Conclusions

The PCR and real-time PCR experiments described above did not reveal any compromise in the quality of results (such as strand breaks brought about by UV irradiation), even after multiple measurements of the DNA samples using the UVette in the UV range of the BioPhotometer. The subsequent use of photometrically measured DNA samples in PCR reactions yielded results comparable to those of unmeasured DNA samples. This result could be verified here for double stranded DNA in two PCR experiments. In the past, we were able to demonstrate the suitability of samples measured in the UVette for subsequent applications using labeled single stranded DNA in microarray experiments [2]. Based on these results, DNA samples whose concentrations were determined in the BioPhotometer plus with the help of the UVette can be used for subsequent PCR experiments.

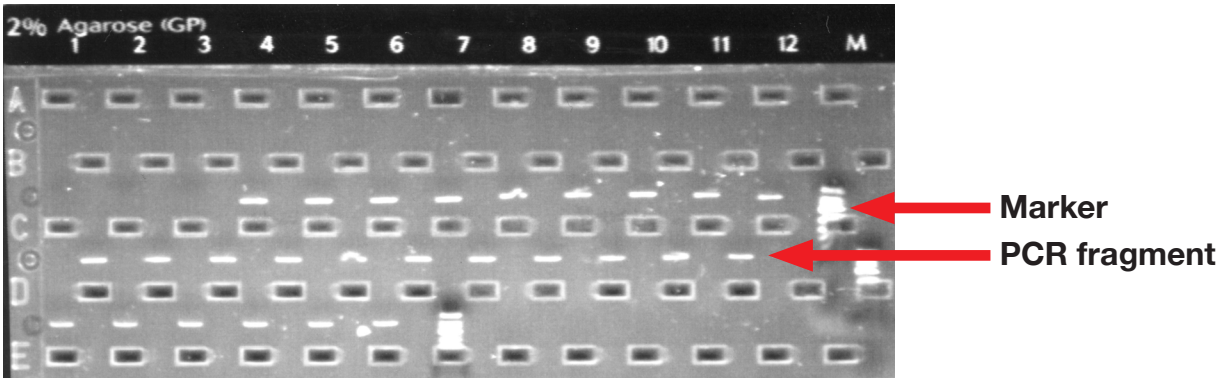

Figure 3: Result of the PCR run
A1-A3 Negative controls, A4-A6 umeasured, high DNA concentration, A7-A9 measured 5 times, high DNA concentration, A10-A12 measured 10 times, high DNA concentration, AM standard, B1-B3 unmeasured, medium DNA concentration, B4-B6 measured 5 times, medium DNA concentration, B7-B9 measured 10 times, medium DNA concentration, B9-B11 unmeasured, low DNA concentration, BM Marker, C1-C3 measured 5 times, low DNA concentration, C4-C6 measured 10 times, low DNA concentration, C7 standard.
No significant differences between the PCR products were observed.

Literature

[1] Cornel Mülhardt: Der Experimentator: Molekularbiologie/Genomics, 4.Auflage, Spektrum Akademischer Verlag (2003) [The Experimenter: Molecular Biology/Genomics, 4th edition; German]

[2] Ludwig Eichinger and Lorna Moll, Martin Armbrecht, Use of Cy labeled cDNA in microarray analyses after determination of the incorporation rate with the Eppendorf BioPhotometer plus and UVettes. Application Note 188. www.eppendorf.com

Ordering information

Designation	Description	Order no. International	Order no. North America
Eppendorf BioPhotometer plus™		6132 000.008	952000006
Thermal Printer DPU 414	serial printer incl. power supply unit and printer cable	6131 011.006	952010140
Printer Paper	5 rolls	0013 021.566	952010409
UVette®	Original Eppendorf plastic cuvettes, individually wrapped, Usable directly in the BioPhotometer, certified RNase-, DNA and protein free, pack of 80	0030 106.300	952010051
UVette® routine pack	Eppendorf Quality purity, re-sealable box, Pack of 200	0030 106.318	952010069
Cuvette rack	For 16 cuvettes	4308 078.006	940001102
BioPhotometer Data Transfer Software		6132854.007	952000500
Secondary UV-VIS-Filter	Test filter set for verification of photometric and wavelength accuracy (in accordance with NIST)	6131 928.007	952010221
ep*Motion*® 5075 LH	PC-Version	5075 000.750	960020101
TS-50 single channel tool	for the volume range 1-50 µL	5280 000.010	960020101
epT.I.P.S.® Motion pipette tips	50 µL volume range, 1-50 µL, 15 x 96 Tips in racks	0030 003.942	960050002
Safe-Lock reaction tubes	2,0 mL, 1,000 pc., colorless	0030 120.094	022363352
Thermorack for 24 x Safe-Lock tubes 0.5/ 1.5/ 2.0 mL Vessels	for preparation of 24 reaction tubes	5075 771.004	960002075
Thermoblock for PCR	96 wells	5075 766.000	960002083
Eppendorf Mastercycler® pro		6321 000.515	950040015
Eppendorf Mastercycler® ep realplex2		6300 000.507	950020202
Eppendorf twin.tec® PCR Platte 96	semi-skirted, 25 pc. (colorless)	0030 128.575	951020303

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