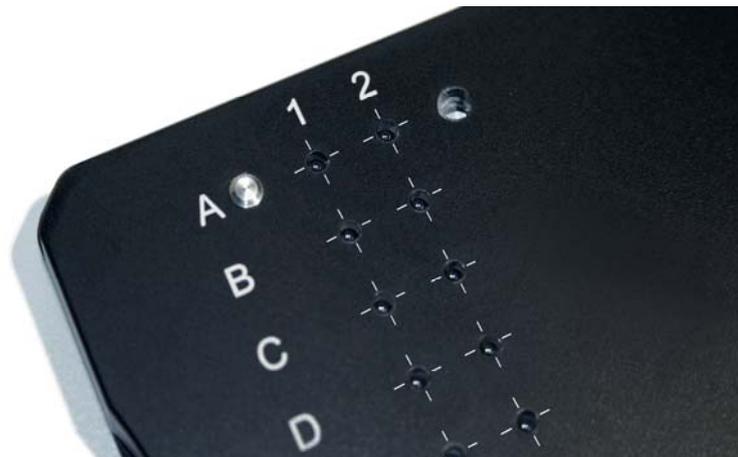
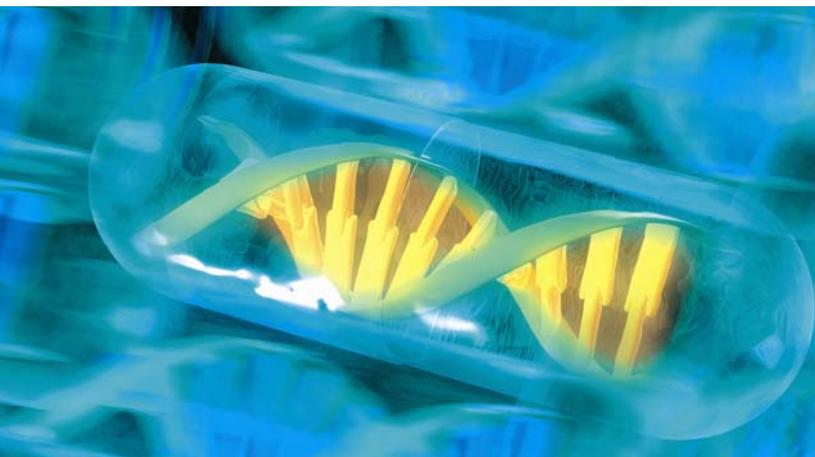


Fluorescence-Based DNA Quantification in Small Volume Samples

Applicability of the NanoQuant Plate™ for Fluorescence Measurements



Introduction

Several procedures in molecular biology require the accurate quantification of DNA. These procedures include cDNA synthesis for the production of libraries, fragment purification and quantitation of amplification products. Concentrations of nucleic acid samples are conventionally determined using absorbance measurements at 260 nm (A_{260}). However, this method is limited in terms of sensitivity and does not discriminate between DNA and RNA. As an alternative, fluorescent dyes that bind to DNA molecules may be used to quantify nucleic samples with a higher sensitivity.

The Infinite® 200 NanoQuant is a fully equipped spectrophotometer with the capacity to perform absorbance measurements in microplates and to easily and accurately quantify small-volume nucleic acid samples using the specifically designed NanoQuant Plate™. In addition, the Infinite 200 NanoQuant can be upgraded at individual convenience with fluorescence reading capabilities enabling the analysis of fluorescent samples as small as 2 µl [1]. All instruments of the Infinite 200 series are capable of performing measurements with the NanoQuant Plate.

This note describes the implementation of the Infinite 200 multimode reader and the associated NanoQuant Plate for fluorescence-based DNA quantification in small volume samples using Pico Green®, an ultra-sensitive fluorescent dye for the quantitation of double-stranded DNA.

Material and Methods

Instrument and plates

- Infinite M200 Quad4 monochromator detection system (Tecan, Austria)
- NanoQuant Plate with 16 individual sample positions with integrated quartz optics (Tecan, Austria)
- 96-well flat bottom polystyrol microplates (Greiner BioOne, Germany)

Reagents and additional materials

- Quant-iT PicoGreen quantitation reagent (Invitrogen, CA, USA)
- Lambda DNA standard, 100 µg/ml (Invitrogen, CA, USA)
- 20x Tris/EDTA buffer (Sigma)
- ddH₂O
- 70% ethanol
- laboratory kimwipes
- single- and multichannel pipettes with suitable tips

Reagent preparation

Tris/EDTA (TE) buffer was diluted from 20x to 1x in ddH₂O. 1x TE was used to dilute both the Quant-iT PicoGreen reagent and the Lambda DNA standard.

The Quant-iT PicoGreen quantitation reagent was prepared freshly as a 200-fold dilution of the stock solution according to the manufacturer's instructions.

Of each dilution 50 μ l were transferred into a 96-well microplate and mixed with 50 μ l of the PicoGreen solution to reach a final volume of 100 μ l. A blank was included, consisting of 50 μ l TE buffer with PicoGreen quantitation reagent.

DNA dilution series

The Lambda DNA standard was diluted from 1000 to 0.9 ng/ml using 1x TE buffer as summarized in table 1.

| Sample | DNA concentration [ng/ml] | DNA concentration per 2 μ l (NQP) |
|--------|---------------------------|---------------------------------------|
| 1 | 1000 | 2.0 ng |
| 2 | 500 | 1.0 ng |
| 3 | 250 | 0.5 ng |
| 4 | 125 | 0.25 ng |
| 5 | 62.5 | 0.12 ng |
| 6 | 31.3 | 0.06 ng |
| 7 | 15.6 | 0.03 ng |
| 8 | 7.8 | 0.015 ng |
| 9 | 3.9 | 0.007 ng |
| 10 | 1.9 | 0.003 ng |
| 11 | 0.9 | 0.002 ng |
| 12 | 0 (blank) | 0 |

Table 1: Dilution series of standard Lambda DNA in TE buffer

Instrument settings

For fluorescence measurements, the NanoQuant Plate has to be used in standard i-control™ mode.

Prior to all measurements, the NanoQuant Plate was cleaned according to the corresponding Quick Guide instructions [1], using an ultrasonic bath and high-pressure compressed air. A measurement script was set up in i-control V1.5 according to the parameters listed in table 2.

| Measurement settings on Infinite M200 NanoQuant | |
|---|---------------------------------------|
| Parameter | Setting |
| Plate | Tecan 16 Flat Black [NanoQuant Plate] |
| Part of the plate | A1-H2 |
| Fluorescence | Top reading mode |
| Excitation | 485 nm |
| Bandwidth | 9 nm |
| Emission | 535 nm |
| Bandwidth | 20 nm |
| Gain | optimal |
| Flashes | 25 |
| Integration time | 20 μ s |

Table 2: Measurement parameters and instrument settings on Infinite M200 NanoQuant with Tecan i-control™ software

Of each DNA dilution that had been mixed 1:1 with the PicoGreen quantitation reagent 2 μ l were transferred from the microplate well onto the samples spots of the NanoQuant Plate. The lid of the plate was then closed carefully and the plate was measured with the script summarized in table 2. All measurements were performed with at least three independent sample replicates.

Results

Linearity

All raw values were blank-corrected and average values, standard deviations (stdev) and CVs were calculated using Microsoft Excel. The average values were plotted in a line diagram and a trend line was added. Slope and coefficient of determination (R^2) were calculated. NQP measurements of Lambda DNA dilutions ranging from 1000 to 0.9 ng/ml show good linearity values, resulting in an R^2 value of 0.9966.

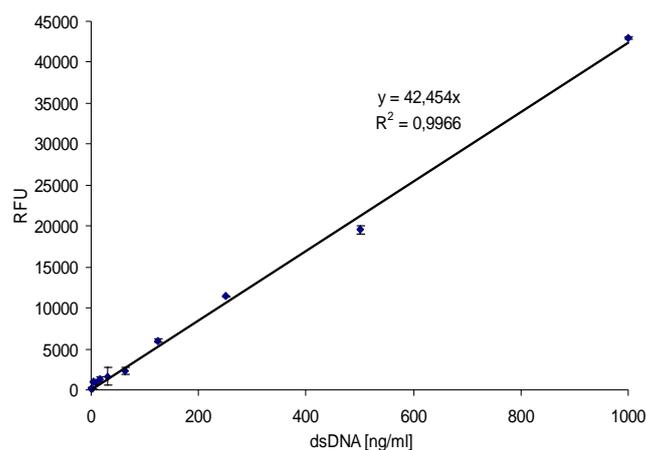


Figure 1: Linearity of fluorescence signals from DNA dilutions

Sensitivity

| DNA concentration [ng/ml] | average RFU | stdev | CV [%] |
|---------------------------|-------------|--------|--------|
| 1000 | 44285 | 40.31 | 0.09 |
| 500 | 20821 | 353.55 | 1.70 |
| 250 | 12691 | 41.72 | 0.33 |
| 125 | 7260 | 376.89 | 5.19 |
| 62.5 | 3636 | 323.15 | 8.89 |
| 31.3 | 2935 | 114.55 | 3.90 |
| 15.6 | 2615 | 169.00 | 6.46 |
| 7.8 | 2194 | 66.47 | 3.03 |
| 3.9 | 2247 | 21.92 | 0.98 |
| 1.9 | 1437 | 152.74 | 10.63 |
| 0.9 | 1479 | 224.15 | 15.15 |
| 0 (blank) | 1281 | 132.94 | 10.38 |

Table 3: Average RFU and stdev of triplicate DNA dilutions from 1000 to 0.9 ng/ml stained with PicoGreen

The sensitivity of PicoGreen-based DNA quantification was calculated using the signal of the 250 ng/ml sample, which was chosen as a representative DNA concentration. The detection limit was calculated according to the below formula:

$$\text{Detection Limit [ng / ml]} = \frac{3 * stdev_{blank} * [c]_{sample}}{(av\ signal - av\ blank)}$$

The resulting detection limit is 8.7 ng/ml dsDNA. This corresponds to approximately 17 pg dsDNA in a sample volume of 2 µl as it is used in the NanoQuant Plate.

| Sample | detection limit per ml | detection limit per 2 µl (NQP) |
|-----------|------------------------|--------------------------------|
| 250 ng/ml | 8.7 ng | 17 pg |

Table 4: Detection limit of PicoGreen-based DNA quantification using the NanoQuant Plate

Discussion

Accurate detection and sensitive quantification of nucleic acids in samples is the basis for many types of applications in molecular biology.

The Quant-iT PicoGreen system in combination with the small volume measurement properties of the NanoQuant Plate represents a sensitive and reliable method for the quantification of dsDNA amounts as small as 17 pg. This is even lower than the quoted detection limit of the Quant-iT PicoGreen kit which is specified to be 25 pg in an assay volume of 200 µl.

In this context, the capability of the NanoQuant Plate to analyse sample volumes of as little as 2 µl even improves the detection range of the Quant-iT PicoGreen system, making it even more sensitive than specified.

Conclusion

The use of the Infinite 200 in combination with the NanoQuant Plate for fluorescence-based quantification of dsDNA offers a time-saving and reliable method to obtain highly sensitive and accurate results with minimal consumption of sample.

Abbreviations

| | |
|-------|-------------------------------|
| cDNA | complementary DNA |
| CV | coefficient of variation |
| dsDNA | double-stranded DNA |
| NQP | NanoQuant Plate™ |
| RFU | relative fluorescence unit(s) |
| RNA | ribonucleic acid |
| ssDNA | single-stranded DNA |
| stdev | standard deviation |
| TE | Tris/EDTA |

Literature

- [1] Quick Guide NanoQuant Plate™, 2008 (Tecan Austria)
- [2] Singer VL et al.: *Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation*. Analytical Biochemistry, 249, 228 - 238 (1997)

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Singapore +65 644 41 886 **Spain** +34 93 490 01 74 **Sweden** +46 31 75 44 000 **Switzerland** +41 44 922 89 22 **UK** +44 118 9300 300
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