Application Brief

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Microplate-Based Spectrophotometric Quantification of dsDNA Using a Variety of Methods

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Introduction

DNA is used for a variety of downstream applications ranging from PCR to sequencing. Standard methods of DNA quantification postisolation include spectrophotometric absorption at 260 nm and fluorescence when DNA is chelated with a binding dye. The most common method remains spectrophotometry due to cost and ease of use. Traditionally, measurements have been done in a cuvette-based spectrophotometer with a 1 cm path length vessel. A microplate format is preferred for analysis of a large number of nucleic acid samples.

Methods

UV absorption

All double-stranded DNA (dsDNA) standards were created by preparing a 1:2 serial dilution series of a concentrated stock in TE buffer (tris-EDTA, pH = 7.0). Measurements were performed in triplicate in a 100 µL volume.

Fluorescence

Herring sperm DNA (part number D6898) was purchased from Sigma-Aldrich (St. Louis, MO). Quant-iT PicoGreen Reagent (part number P11495) was purchased from Thermo Fisher Scientific (Waltham, MA). Solid 96-well black microplates (part number 3915) were from Corning Inc. (Corning, NY). Herring sperm DNA was diluted to 2 µg/mL with TE (10 mM Tris, 1 mM EDTA, pH 7.5) as the diluent. The final concentration was confirmed using 260 nm absorbance. A series of dilutions ranging from 0.0 to 2,000 ng/mL of purified herring sperm was made using TE and 100 µL aliquots pipetted into microplate wells. Equal amounts (100 µL) of working PicoGreen quantitation reagent were mixed and incubated for 10 minutes at room temperature, protected from light. Working PicoGreen reagent was prepared by diluting the concentrated DMSO-PicoGreen stock solution 1:200 with TE according to the manufacturers' recommendations.

Instrumentation

UV absorption

The Beer-Lambert Law relates the absorption and concentration via the following equation where A = log Io/I = εlc. ε refers to the extinction coefficient of the analyte, l is the pathlength (cm) and c is the concentration of the analyte (ng/µL).

The Agilent BioTek Gen5 microplate reader and imager software has built-in methods for path length correction to 1 cm for samples in microplates where path length is defined by the volume of the sample added. The calculation is performed by measuring the absorbance peak of water at 977 nm and 900 nm; the difference is then divided by 0.18, which is the absorbance of water at 1 cm, equaling the sample's path length. The Agilent BioTek Synergy LX multimode reader with absorbance monochromators was used to measure absorbance at 230, 260, 320, 900, and 977 nm in a single protocol to allow automated path length correction, DNA quantification, and ratiometric sample purity assessment.

Fluorescence

Fluorescence was determined using the Synergy LX. The onboard software and touch-screen interface were used to select the appropriate filter cube, define shaking parameters, add delays, and select well locations and protocol parameters. A GFP filter cube (Ex 485/20 nm, Em 528/25 nm and 510 nm cutoff dichroic mirror) was used for all measurements. Auto gain was selected with Extended Dynamic Range. A 30-second orbital shake was performed followed by a 10-minute delay for incubation at room temperature. Alternatively, the instrument can be controlled and analysis performed in the Gen5 software or data imported for analysis.

Results and discussion

The use of absorbance and fluorescence methods for quantification of double-stranded DNA allows a wide range of concentrations to be analyzed. UV absorbance is the most common and easiest method requiring no additional reagents but can suffer from limits of detection especially when working with submicrogram-per-milliliter DNA samples. Figure 1 shows good linearity to the low µg/mL concentrations of DNA. The data indicate a limit of detection of ~8 µg/mL using UV absorbance or ~0.8 µg in the 100 µL volume.

The ability to take multiple UV absorbance readings at different wavelengths provides a means to verify sample purity as well. Typically, the ratio of A_{260}/A_{280} is used with values between 1.8 to 2.0, indicative of a pure sample. It is common that for very low concentration samples the ratio is generally lower than the expected value due to a greater influence of the A_{280} measurement in the calculation (Table 1).

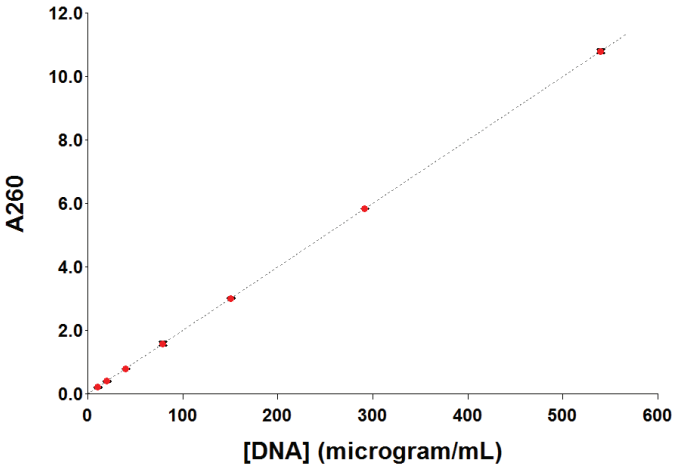

Figure 1. Absorbance measurements. A dilution series of herring sperm DNA was prepared ranging from 0 to 550 µg/mL in TE buffer. Standards were analyzed in triplicate at a volume of 100 µL in a standard 96-well microplate format. A_{260} measurements are path length corrected.

Table 1. A_{260}/A_{280} ratio. The ratio of absorbance measurements at 260 and 280 nm can be used to access the purity of a sample. Typical ratios for pure DNA range from ~1.8 to 2.0.

[DNA] (µg/mL)	A_{260}/A_{280}
540	1.8
291	1.9
151	1.8
79	1.8
40	1.7
20	1.6
10	1.5

The use of fluorescence methods can extend the range of quantification to picogram quantities of DNA in a standard microplate assay format. Figure 2 shows the ability to measure low ng/mL quantities of DNA. The data indicate a limit of detection of ~121 pg/mL using fluorescence or ~12.1 pg in the 100 µL volume.

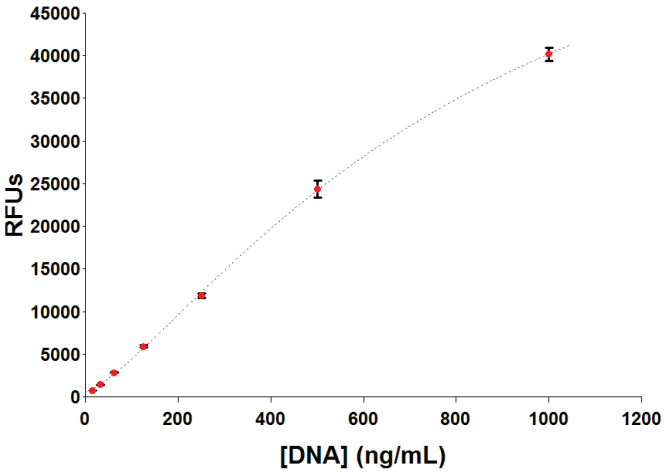

Figure 2. Fluorescence assay. A dilution series of herring sperm DNA was prepared ranging from 0 to 1,000 ng/mL in TE buffer. Standards were analyzed in triplicate in a standard 96-well microplate format.

Conclusion

The Agilent BioTek Synergy LX multimode reader provides the most common detection technologies used in biological research including absorbance, fluorescence, and luminescence detection. This application brief demonstrated its utility to quantify DNA in microplates using intrinsic UV detection and a fluorescence assay to extend the dynamic range of measurable concentrations.

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