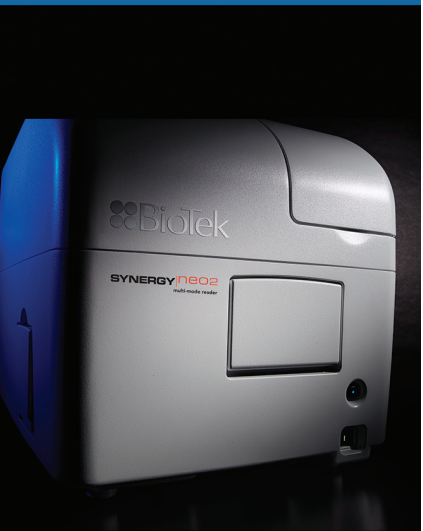


Fluorometric Quantitation of dsDNA using PicoGreen®

Using Synergy™ Neo2 Multi-Mode Reader to Quantify DNA in Solution

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DNA
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Abstract

An essential element of cellular and molecular biology is the ability to quantitate DNA in large numbers of samples at a sensitivity that enables determination of small amounts of sample. Here we describe a method to quantify dsDNA using BioTek's Synergy™ Neo2 Multi-Mode Reader.

Introduction

Many techniques of cellular and molecular biology require the ability to quantify dsDNA in large numbers of samples at sensitivities that only require a small amount of the total sample. Isolation of plasmids from bacterial cultures, genomic DNA from mammalian cells, cDNA synthesis for library production, and quantitation of PCR products for diagnostic purposes all require the direct quantitation of dsDNA. Also, many biochemical studies that involve the growth kinetics of cell cultures or cell cycle studies require normalization by DNA content.

The most commonly used method for the determination of nucleic acid concentration is the determination of absorbance at 260 nm (A_{260}) as described by Matiatis et al.^[1]. This method, while quite adequate for many situations, can suffer from the interfering absorbance of contaminating molecules. Many of these contaminants which include nucleotides, RNA, EDTA and phenol are commonly found in nucleic acid preparations. As a result, several fluorescent staining techniques have been developed to alleviate many of the problems associated with absorbance at 260 nm^[2-4]. One such stain is PicoGreen®, which in conjunction with BioTek's Synergy™ Neo2 Multi-Mode Reader offers high specificity, as well as, high sensitivity for dsDNA quantitation.

Materials and Methods

Quant-iT™ PicoGreen Reagent, cat # P11495, Salmon Sperm DNA (cat # 15632011) and TE (100x) buffer concentrate (cat # BP-1338-1) were purchased from Thermo-Fisher. Solid 96-well black microplates, (cat # 3915), were from Corning.

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 2000 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, provided in the PicoGreen kit, 1:200 with TE according to the kit instructions^[2]. Following incubation, the fluorescence was determined using a Synergy Neo2 Multi-Mode Reader. With filter-based measurements, the reader used a 485 nm, 20-nm bandwidth, excitation filter and a 528 nm, 25-nm bandwidth emission filter along with a 510 nm cutoff dichroic mirror. Monochromator-based determinations were made with an excitation wavelength of 485 nm and an emission wavelength of 528 nm, with the reader default bandwidth. The reader was programmed to "Autoscale" the sensitivity setting to the highest DNA concentration. The data was collected from the top using a 0 msec second delay after plate movement, 25 reads per well. Emission spectral scans were made by fixing the excitation at 485 nm (9 nm bandwidth) and varying the emission wavelengths in 1 nm increments. Excitation scans were produced by fixing the emission wavelength at 528 nm and varying the excitation wavelengths in 1 nm increments. All bandwidths were set to 9 nm and the PMT gain setting set at 125 using normal read speed. Reactions were typically determined within one hour.

Results

Fluorescent spectral scans of dsDNA (1 $\mu\text{g}/\text{mL}$) stained with PicoGreen reagent demonstrates excitation and emission peaks at 485 nm and 528 nm respectively after blank subtraction (Figure 1). These wavelengths were then used for subsequent measurements in order to maximize fluorescence signal for DNA titration experiments.

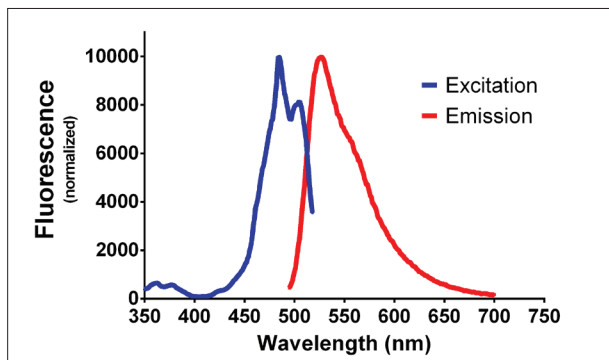


Figure 1. Fluorescent excitation and emission spectrum of dsDNA (1 $\mu\text{g}/\text{mL}$) stained with PicoGreen reagent. Data was normalized such that maximum value was 10,000 RFUs.

The fluorescence intensity was determined for DNA concentrations ranging from 0 to 1000 ng/ml. Over this range the intensity increased in a linear fashion. Using Gen5™ data reduction software (BioTek Instruments), a least means squared linear regression analysis can be generated with a coefficient of determination (r^2) value of 0.998. The average coefficient of variance (%CV) of the standards was less than 3%, with the greatest variation in the lower DNA concentrations tested (data not shown). In terms of sensitivity, the assay was found to be sensitive to the picogram level. Under appropriate sensitivity settings, DNA concentrations as low as 30 pg/mL were found to be statistically different ($P < 0.05$) from the TE only, 0 ng/mL, control. Quantitation of dsDNA using the fluorescent properties of PicoGreen in conjunction with the Synergy™ Neo2 allows researchers to quantitate as little as 6 pg/well (30 pg/mL in a 0.2 mL total volume); thus providing reliable quantitation of dsDNA concentrations ranging over four orders of magnitude.

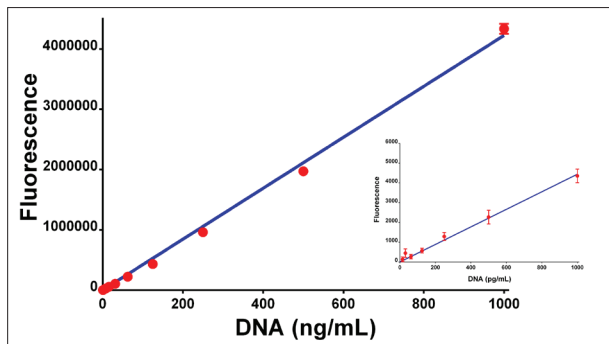


Figure 2. Linearity of Spectral Filter Based Detection. Fluorescence of dsDNA titration incubated with PicoGreen reagent was made using a 485/528 Synergy Neo filter cube. Concentration ranged from 0.0 to 1 $\mu\text{g}/\text{mL}$ of dsDNA with least means squared linear regression analysis. Insert figure depicts the data points for the lower concentrations (0 to 1000 pg/mL). Data points represent the mean values of eight determinations at each concentration.

In addition to filter cubes for fluorescence detection, the Synergy Neo2 can be configured with dual monochromators for both excitation and emission. Similar linearity can be observed using the monochromators for wavelength selection as compared to the filter based detection (Figure 3). Similar detection limits (30 pg/mL) were observed with the monochromators as with the filter based optical system.

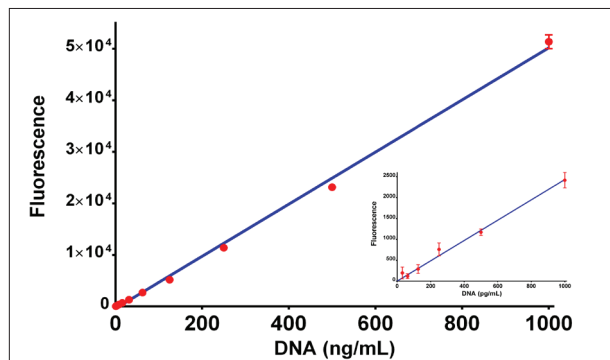


Figure 3. Linearity of Monochromator Based Detection. Fluorescence of dsDNA titration incubated with PicoGreen reagent was made using a Synergy Neo 2 monochromators. Excitation was set to 485 nm and the emission was set to 528 nm, with a bandwidth of 20 nm for both excitation and emission. Concentration ranged from 0.0 to 1 $\mu\text{g}/\text{mL}$ of dsDNA with least means squared linear regression analysis. Insert figure depicts the data points for the lower concentrations (0 to 1000 pg/mL). Data points represent the mean values of eight determinations at each concentration.

Despite the lower magnitude in raw scale, the monochromators provide a better signal to background ratio as compared to the filters, as demonstrated in Figure 4. This is primarily the result of lower background measurements produced by the dual monochromators.

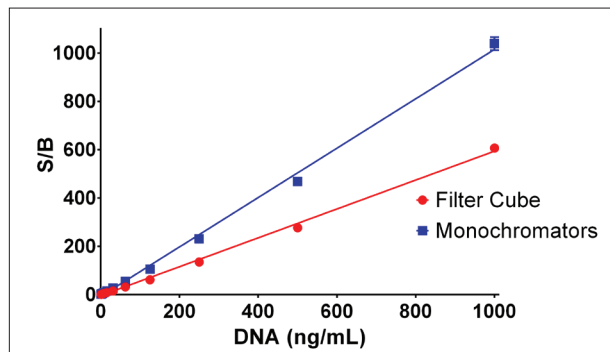


Figure 4. Comparison of signal to background ratios of monochromator and filter based optical paths. The ratio of each sample to the mean of the blank (0 $\mu\text{g}/\text{mL}$ dsDNA) was plotted. Data represents the mean and standard deviation of eight determinations.

The Synergy Neo2 Multi-Mode Reader is a highly configurable multi-mode microplate reader. In this treatise we have examined the filter based and monochromator based fluorescence detection optical systems. Filter based detection is provided with an array of predefined filter cubes that have barcode identification. This allows the reader to insure that the correct filter combination is used for the intended fluorescent moiety. The spectral characteristics of the filters and dichroic mirrors employed maximize the excitatory light on target as

well as the recovery of the emitted signal, maximizing sensitivity. The dual monochromator system, while resulting in lower raw signal than the filter system, offers complete flexibility in regards to wavelength selection as well as bandwidth. In addition, the high resolution design of the monochromators results in very low background fluorescence, which translates into better Signal to Blank (S/B) ratios than the filter-based optics.

In order to achieve optimal sensitivity, several measures can be undertaken. Removal of certain common contaminants, such as proteins or organics (e.g. phenol and butanol), will result in tighter CVs and lower background signal, resulting in an increase in sensitivity. Several compounds are known to decrease the fluorescent signal when present in a PicoGreen-DNA assay. In particular, the presence of 200 nM sodium chloride or 50 mM magnesium chloride can decrease the signal by approximately 1/3. In cases where only low DNA concentrations are to be determined, a calibration curve utilizing lower concentrations can be used. Additionally, the use of microplates that utilize lower fluorescent plastics would be expected to reduce background and therefore increase sensitivity.

References

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