



Quantitation of DNA using Hoechst 33258

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 fluorescent method to quantitate DNA with Hoechst 33258 dye and the BioTek FL600 fluorescence microplate reader. This assay provides linear results over three orders of magnitude and can routinely detect 8 ng of DNA. Reduction of the dye concentration was found to improve sensitivity to as low as 600 pg/well.

Introduction

Many techniques of cellular and molecular biology require the ability to quantitate dsDNA in large numbers of samples at sensitivities that only require a small amount of the total sample. For example, cycle sequencing reactions require that appropriate concentrations of bacterial miniprep DNA are used in order to be successful on a consistent basis. Many biochemical studies that involve the growth kinetics of cell cultures or cell cycle studies require normalization by DNA content.

Although there are many different methods to quantitate DNA, most methods have disadvantages that preclude their use in many applications. Absorbance measurements at 260 nm (A₂₆₀) is the most commonly used method for DNA concentration determination, but it suffers from the interfering absorbance of contaminating molecules (1). Many of these contaminants, which include nucleotides, RNA, EDTA and phenol, are commonly found in nucleic acid preparations. The fluorescent bisbenzimidazole (Hoechst) dyes circumvent many of these problems. Hoechst 33258 dye is relatively selective for dsDNA and in high salt does not show fluorescent enhancement in the presence of either protein or RNA. The dye, weakly fluorescent itself in solution, binds specifically to the A-T base pairs in dsDNA resulting in an increase in fluorescence and a shift in the emission maximum from 500 to 460 nm (2, 3). The use of Hoechst 33258 in conjunction with the BioTek FL600 fluorescence microplate reader offers high specificity, as well as high sensitivity for dsDNA quantitation.

Materials and Methods

Bisbenzimidazole (Hoechst 33258), catalogue number B-2883, was purchased from Sigma Chemical Co., St. Louis, Missouri, as were sodium chloride and sodium monobasic phosphate. The 96-well black microplates with clear bottom, catalogue number 3603, were purchased from Costar, (Cambridge, MA). Low fluorescent background black flat-bottom plates, catalogue number 011-010-7805, were obtained from Dynatech Laboratories, Inc. (Chantilly, VA). Assay buffer (2M NaCl, 50mM NaH₂PO₄, pH 7.4) was previously prepared and sterilized by autoclaving and stored at 4°C. Prior to use a portion of the buffer was allowed to warm to room temperature. Hoechst dye stock (1 mg/ml in distilled H₂O) was previously prepared and sterilized by filtration through a 0.22 µm filter and stored at 4°C in a light tight container. Working assay solution was prepared fresh prior to each assay by mixing 1 ml of concentrated dye stock solution for every 1 ml of assay buffer required resulting in a final Hoechst 33258 concentration of 1 mg/ml. Working

assay solutions containing final Hoechst dye concentrations of 0.1 mg/ml and 0.01 mg/ml were prepared by diluting the 1.0 mg/ml solution with assay buffer 1:10 and 1:100 respectively.

Dilutions of sonicated herring sperm DNA were made using the three working assay solutions, each containing different dye concentrations, as the diluent. After the dilutions were made, 200 µl aliquots were pipetted into microplate wells in replicates of six. Fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader with a 360 nm, 40 nm bandwidth, excitation filter and a 460 nm, 40 nm bandwidth emission filter. The data collected from either the top or the bottom using static sampling with a 0.35 second delay, 100 reads per well at several differing sensitivity settings as required by experimental conditions. Although in these experiments the plates were read immediately, if they remained sealed and protected from light the reaction was found to be stable for several hours.

Results

The fluorescence intensity was determined for DNA concentrations ranging from 0 to 20 mg/ml (figure 1). Over this range, the intensity increased in a linear fashion.

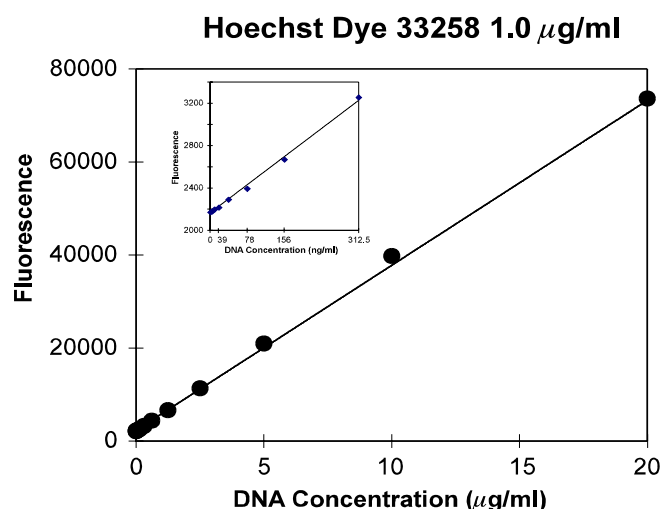


Figure 1. Linearity of the assay at standard bisbenzimidazole dye concentrations. Concentration curve from 0.0 to 20 mg/ml with linear regression analysis. Insert figure depicts the data points for the lowest DNA concentrations (0.0 to 312.5 ng/ml). Filled circles (●) represent the mean values of eight determinations at each concentration. Fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader with the sensitivity setting at 40 and the data collection from the bottom using static sampling with a 0.35 second delay, 100 reads per well.

Using Microsoft® Excel a least means, squared linear regression analysis can be generated with a coefficient of determination (r^2) value of 0.999. The average coefficient of variation (%CV) of the standards was less than 2%, with the greatest percent variation-taking place in the lower DNA concentrations tested (data not shown). In terms of sensitivity, the assay was found to be sensitive to the nanogram level. Under appropriate sensitivity settings, DNA concentrations as low as 40 ng/ml were found to be statistically different ($p=0.005$) from the working assay solution only, 0 ng/ml DNA, control. Quantitation of dsDNA using the fluorescent properties of Hoechst dye 33258 in conjunction with the BioTek FL600 allows researchers to quantitate as little as 8 ng/well (40 ng/ml in a 0.2 ml total volume). Using the described conditions, the assay was found to be linear over three orders of magnitude.

In order to achieve greater sensitivity a number of measures can be undertaken to reduce background fluorescence. The use of different microplates that utilize low fluorescent plastics would be expected to reduce background and therefore increase sensitivity. Likewise, the reduction of background dye fluorescence would also be expected to increase sensitivity.

Because of the inherent background fluorescence of bisbenzimidazole in solution, the reduction of Hoechst 33258 dye concentrations to below 1 mg/ml has been suggested as a means of decreasing background fluorescence and thus improving the detection of lower DNA concentrations (3). In order to optimize our detection limits, we sought to examine the effect of dye concentration on the fluorescent signal obtained over a range of DNA concentrations.

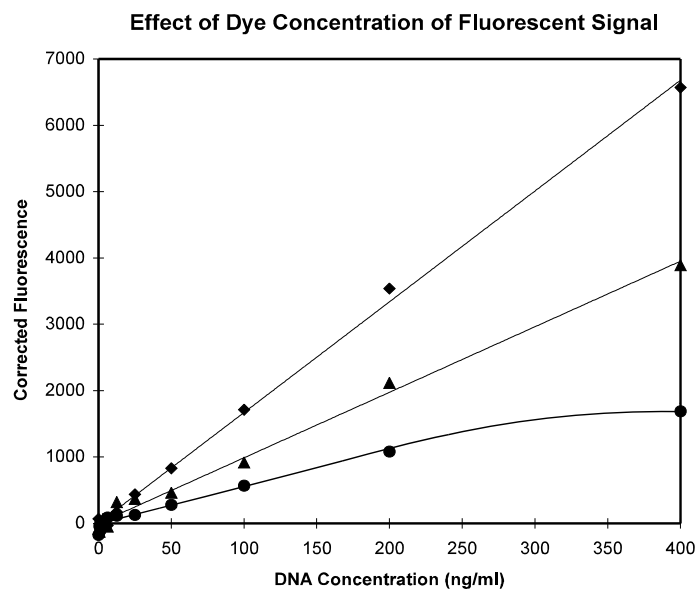


Figure 2. The Effect of Hoechst 33258 Dye Concentration on Fluorescent Signal. Dilutions of DNA were made with three different concentrations of Hoechst 33258 dye (u 1.0 $\mu\text{g/ml}$; s 0.1 $\mu\text{g/ml}$; l 0.01 $\mu\text{g/ml}$) and their fluorescent signal determined. For each dye concentration fluorescent signal was corrected by subtracting the average response of the 0 ng/ml DNA concentration from all the determinations. In all cases the fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader with the sensitivity setting at 74 and the data collection from the top using static sampling with a 0.35 second delay, 100 reads per well.

The fluorescent intensity was determined for DNA concentrations ranging from 0 to 400 ng/ml using three different concentrations of bisbenzimidazole dye. Figure 2 demonstrates that decreasing dye concentrations result in lower fluorescent signals for a given DNA concentration even after correction for differing baseline fluorescent signals. Dye concentrations of 1.0 $\mu\text{g/ml}$ and 0.1 $\mu\text{g/ml}$ both produce a linear response with respect to DNA concentrations from 0 to 400 ng/ml, with coefficient of determination values indicating a high degree of confidence ($r^2=0.97$ and $r^2=0.98$ respectively). Further dilution of the dye concentration to 0.01 mg/ml results in a hyperbolic shaped curve indicating that for DNA levels above 200 ng/ml the bisbenzimidazole dye is no longer in excess. From the data we decided that reducing the bisbenzimidazole dye concentration to 0.1 mg/ml would result in lower background fluorescence, yet provide adequate amounts of dye to provide linearity.

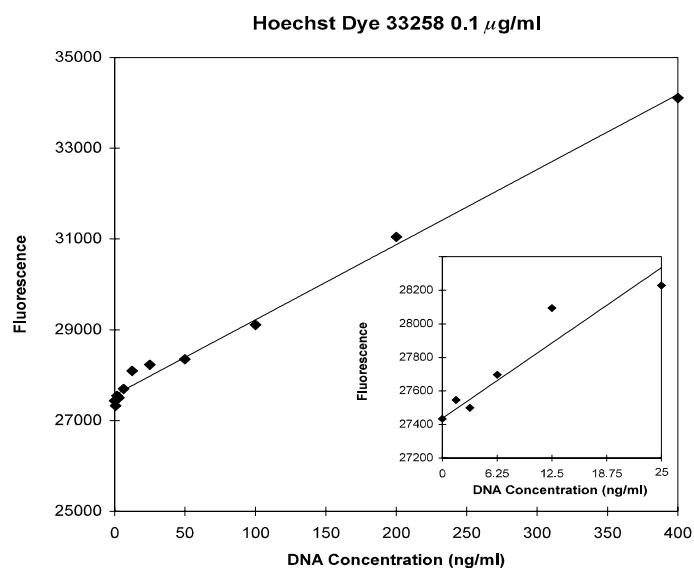


Figure 3. Linearity of the Assay at reduced bisbenzimidazole concentrations. DNA concentration curve from 0.0 to 400 ng/ml made using 0.1 mg/ml Hoechst 33258-dye solution with linear regression analysis. Insert figure depicts the data points for the lowest DNA concentrations (0.0 to 25 ng/ml). Filled diamonds (u) represent the mean values of six determinations at each concentration. In all cases the fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader with the sensitivity setting at 80 and the data collection from the top using static sampling with a 0.35 second delay, 100 reads per well.

As demonstrated in figure 3, a ten fold reduction in dye concentration along with the use of microplates with low background fluorescence result in lower detection limits. In this experiment, using the lower Hoechst 33258 concentration of 0.1 mg/ml reduced the detection limit to 3 ng/ml ($p=0.03$) or approximately 600 pg/well. The average coefficient of variation (%CV) of the standards was still less than 2% (data not shown).

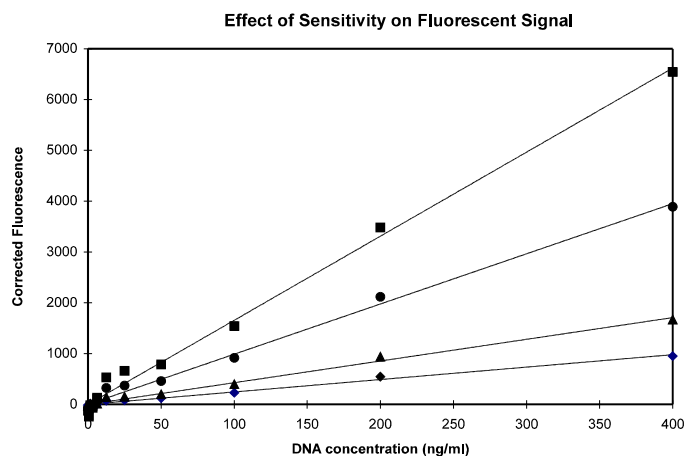


Figure 4. The Effect of Sensitivity setting on Fluorescent Signal. Dilutions of DNA were made with a 0.1 mg/ml Hoechst 33258-dye solution and placed in Microfluor B 96-well fluorescent microplates. For each sensitivity setting (u 60; s 66; l 74; n 80) the fluorescent signal was corrected by subtracting the corresponding average response of the 0 ng/ml DNA concentration from all the determinations. In all cases the fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader. The data collected from the top using static sampling with a 0.35 second delay, 100 reads per well.

Because a low fluorescent signal can be offset by an increase in the sensitivity setting on the FL600, the effect of different sensitivity settings on the fluorescent signal was tested using 0.1 mg/ml Hoechst 33258 dye with DNA concentrations ranging from 0 ng/ml to 400 ng/ml. As shown in figure 4, increasing the sensitivity will increase the fluorescent signal obtained from a

given DNA and bisbenzimidazole dye combination. It is important to note that by correcting each sensitivity setting to zero for the 0 ng/ml DNA sample, any increase in signal due to an increase in the background fluorescence has been eliminated. After correction, the signal at each specific DNA concentration is amplified with an increase in the sensitivity, resulting in divergent increasing values. A simple increase in the baseline fluorescence would result in parallel lines that would be superimposed upon one another after correction.

Discussion

Optimization of assay conditions must be determined empirically, as several factors must be weighed against one another. For most investigators using the conditions described by Labarca and Paigen (3) will be adequate. In fact, using a Hoechst 33258 dye concentration of 1.0 mg/ml would be expected give a linear response to a wider range of DNA concentrations than lower dye concentrations. Alternatively for low levels of DNA the use of 0.1 mg/ml dye concentrations may be more appropriate, as background fluorescence is reduced. The caveat of this condition being a decrease in fluorescent signal response resulting in a flatter dose response. The reduction in fluorescent signal in turn can be compensated for by increasing the sensitivity setting on the FL600 fluorescent plate reader, but in doing so, earlier saturation of the photomultiplier tube results in the loss of ability to quantitate higher levels of DNA. The ability to adjust both the assay and the FL600 allows the user to adjust the assay to meet his or her individual needs.

The ability to perform this assay in microplates offers several advantages over the conventional tube-based fluorescence assays. Like most assays that are performed in microplates, the ability to use multi-channel pipettes greatly reduces the manual labor required to perform the assay. The microplate format also lends itself to "off the shelf" automation for laboratories with high volume requirements. The smaller reaction volumes in microplates will lead to lower per assay costs by reducing the amount of expensive reagents necessary to perform the assay.

References

- (1) Maniatis, T., E.F. Fritsch, and J. Sambrook (1982) Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Springs Harbor, NY.
- (2) Daxhelet, G.A., M.M. Coene, P.P. Hoet, and C.G. Cocito (1989) Analytical Biochemistry 179:401-403.
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Paul G. Held Ph.D.
Senior Scientist & Applications Lab Manager

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