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Application Note

Increasing the Range of DNA Quantitation when using Hoechst Dye 33258

Introduction

The quantitation of DNA is an essential element of cellular and molecular biology. The use of Hoechst dye 33258 is commonly used for this purpose. In instances where very low levels of analyte need to be quantitated, a case can be made for trying to improve detection limits. Alternatively, there are situations where levels of the analyte exceed the normal range of the calibration curve and raising the highest quantifiable concentration is preferable to dilution of the unknown sample. In either situation, there may be a need to increase the dynamic range of the assay's calibration curve rather than switching to another assay with a differing concentration range. The purpose of this report is to demonstrate the utility of altering specific parameters in the bisbenzimide-DNA assay described by Labarca and Paigen (1) to accommodate an increase in the range of DNA concentrations that can be quantified using this assay.

Materials and Methods

Bisbenzimide (Hoechst 33258), catalogue number B-2883, was purchased from Sigma Chemical Co., St. Louis, Missouri as were sodium chloride and sodium monobasic phosphate. Low fluorescent background black flat-bottom Microfluor B plates, catalogue number 011-010-7805, were obtained from Dynatech Laboratories, Inc. (Chantilly, VA). Assay buffer (2M NaCl, 50mM NaH_2PO_4, 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 H_2O) 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. Hoechst 33258 concentration of 10 µg/ml was prepared by mixing 10 µl of stock dye solution for every 1 ml of assay buffer required. Likewise, when a dye concentration of 1 µg/ml was required, 1 µl of stock was mixed for every 1 ml of assay buffer. Working assay solutions containing final Hoechst dye concentrations of 0.01 µg/ml were prepared by diluting the 1.0 µg/ml solution 1 to 100 with assay buffer.

Results

Utilizing the bisbenzimide concentration of 1 µmg/ml recommended by Labarca and Paigen (1) results in a linear dose dependent increase in fluorescent signal with DNA concentrations from 0 to 20 µg/ml (figure 1). Above the 20 µg/ml level the signal slope begins to decrease and is virtually flat above 100 µg/ml. This is true regardless of the sensitivity setting utilized, with only the signal intensity values changing with differing settings.

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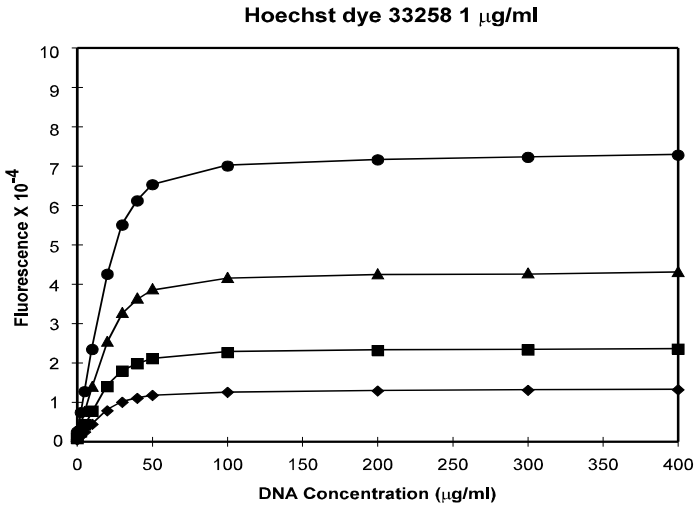

Figure 1. The effect of 1.0 µg/ml Hoechst 33258 dye concentration on fluorescent signal. Dilutions of DNA were made with Hoechst 33258 dye at 1 µg/ml. Fluorescent signal determined at four different sensitivity (u 44; n 48; s 52; l 56) settings and their fluorescent signal determined.

Increasing the Hoechst 33258 concentration 10 fold to 10 µg/ml increases the range of linearity approximately ten fold to 200 µg/ml (figure 2). DNA concentrations from 200 to 400 µg/ml increases fluorescent signal, but the increase is no longer linear. A polynomial regression analysis describing the calibration curve can be used to determine DNA concentrations in this range with a high degree of confidence (figure 2).

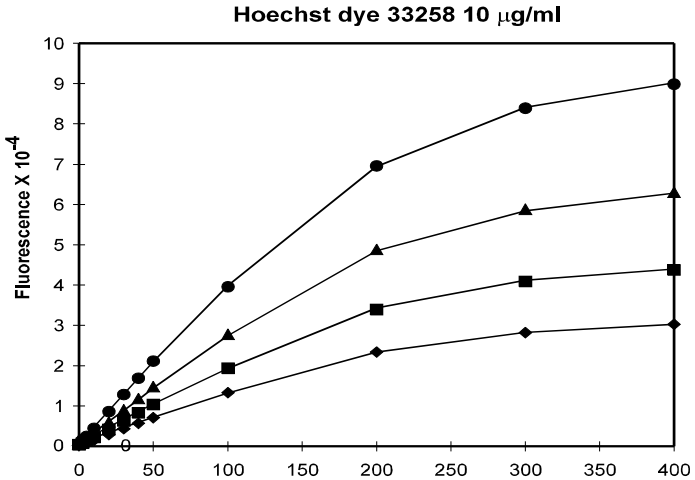

Figure 2. The effect of 10 µg/ml Hoechst 33258 of DNA were made with Hoechst 33258 dye at 10 µg/ml. Fluorescent signal determined at four different sensitivity (u 38; n 40; s 42; l 44) settings and their fluorescent signal determined.

Because of background fluorescence of the compound, it has been suggested that decreasing the bisbenzimide concentration will result in lower detection limits (1). In order to address this we reduced the bisbenzimide concentration 100 fold to 0.01 µg/ml. As expected, the detection limit was reduced from 40 ng/ml using a Hoechst dye concentration of 1.0 µg/ml at to 6.25 ng/ml with a dye concentration of 0.01 mg/ml (data not shown). However, when the reduced dye concentration is used the linear range of the calibration curve is decreased to a maximum DNA concentration of 0.2 µg/ml (figure 3). The DNA detection limit when using a bisbenzimide concentration of 10 µg/ml was determined to be 200 ng/ml (data not shown).

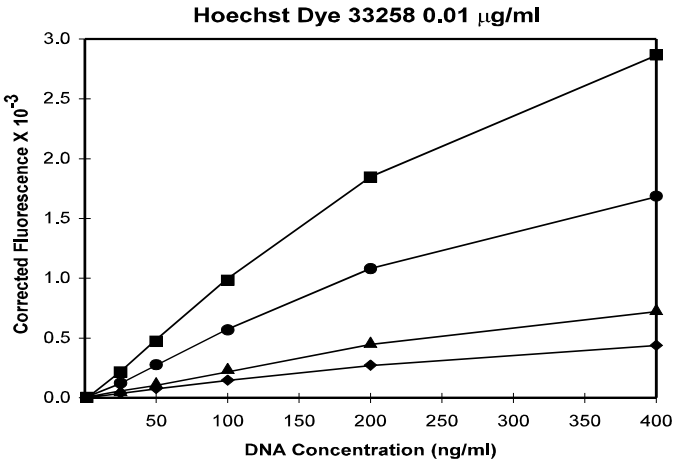

Figure 3. The effect of 0.01 µg/ml Hoechst 33258 dye concentration on fluorescent signal. Dilutions of DNA were made with Hoechst 33258 dye at 0.01 µg/ml. Fluorescent signal determined at four different sensitivity (u 60; s 66; l 74; n 80) settings and their fluorescent signal determined.

Discussion

The dynamic range of quantitation of the bisbenzimide (Hoechst 33258) assay can be increased at either end of the calibration curve. It is clear that increasing the fluorescent dye concentration allows for an increase in the dynamic range of the Hoechst dye 33258-DNA assay. 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 (1, 2). Linearity of the assay is thus based upon an excess of dye relative to dsDNA target. The flattening of the concentration curve seen with the 1.0 µg/ml dye level at high DNA concentrations is most likely the result of limiting amounts bisbenzimide molecules relative to the A-T base pairs and the addition of 10 fold more dye would in turn be expected to alleviate this problem. Similarly, reduction of dye concentrations, in order to reduce background fluorescence, will result in reduction of the maximal DNA concentration that can be quantified at the expense of higher DNA concentrations.

References

(1) Labarca, C. and K. Paigen (1980). Analytical Biochemistry 102:344-352.
(2) Daxhelet, G.A., et al. (1989) Analytical Biochemistry 179:401-403.

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