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Technical Brief

Quantifying DNA concentrations using fluorometry: A comparison of fluorophores

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Detection of low concentrations of DNA is important in vision research because many animal models only provide scant samples of ocular tissue. Quantitative analysis of low concentrations of double stranded DNA is now feasible using fluorometry with newer fluorophores. This technique offers a rapid way to evaluate the DNA content of samples based on the measurement of fluorescence enhancement emitted by fluorophore-bound DNA and is more sensitive than absorption spectrometry. The purpose of this study was to compare the sensitivity of several different fluorophores for measuring DNA concentrations by fluorometry. Based on our studies, we conclude that SYBR Green I and PicoGreen are substantially more sensitive for quantifying DNA concentrations than ethidium bromide and Hoechst 33258.

The determination of DNA concentration using as little DNA as possible is now important in vision research because of the need to analyze small samples of ocular tissue. Quantitative analysis of low concentrations of double stranded DNA (dsDNA) is now feasible using fluorometry with newer fluorophores (fluorescent stains). Fluorometric measurement of DNA concentration has gained popularity because it is simple and potentially much more sensitive than absorbance measurements. This study examined four fluorophores (ethidium bromide, Hoechst 33258, PicoGreen, and SYBR Green I), each with characteristics that may be helpful or a hindrance in different situations.

Ethidium bromide preferentially binds to dsDNA by intercalation. It is both toxic and mutagenic. In contrast, Hoechst 33258 is non-intercalating, apparently binding to the minor groove of DNA with a marked preference for AT sequences [1]. PicoGreen is a fluorophore that selectively binds dsDNA and has characteristics similar to those of SYBR Green I, with similar excitation/emission maxima [2]. Both PicoGreen and SYBR Green I appear to exhibit high affinity for DNA and a large fluorescence enhancement upon DNA binding [2]. The fluorescence enhancement of PicoGreen is exceptionally high; little background occurs since the unbound fluorophore has virtually no fluorescence [2]. PicoGreen is very stable to photobleaching, allowing longer exposure times and assay flexibility [2]. SYBR Green I is a sensitive dsDNA stain, making it useful for many applications where there is a low concentration of DNA, including the detection of low cycle number and low target number DNA amplification products [3]. The capability of SYBR Green I to selectively assay dsDNA in the presence of contaminants (RNA, ssDNA, nucleotides, and protein) is advantageous. Also, the presence of SYBR Green I bound to DNA does not inhibit the activity of some restriction endonucleases, including *Hin*dIII and *Eco*RI [4,5].

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A_{260}) [6]. While this technique is simple and easy, it is inadequate to detect small quantities of dsDNA. The absorbance method does not distinguish nucleotides, single-stranded DNA (ssDNA), some contaminants, and RNA [6]. Moreover the assay is insensitive (typical sensitivity is 150 ng/ml of dsDNA) [7].

Fluorometry is widely regarded as one of the most sensitive molecular techniques for measuring DNA concentrations [8]. Changes in the spectral characteristics of DNA-bound fluorophores permit DNA concentration to be determined by fluorometry. In the present study, we compared the sensitivity and precision of four fluorophores for measuring dsDNA concentrations.

METHODS

Preparation of stock fluorophore solutions: The fluorophores were purchased in solution and diluted based on manufacturer recommendations. A 10 mg/ml stock solution of ethidium bromide (EtBr; Sigma, St. Louis, MO; catalog number E8751) was used. The stock solution was diluted in Tris NaCl-EDTA buffer (TNE; 10 mM Tris base, 0.2 M NaCl, 1 mM EDTA, pH 7.4) to give a concentration of 5 μg/ml of EtBr in the final assay solution. Hoechst 33258 stock solution (Molecular

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Probes Inc., Eugene, OR; catalog number H3569) was diluted 1:400 in TNE buffer to give a concentration of 0.025 mg/ml. PicoGreen stock solution (Molecular Probes, Eugene, OR; catalog number P7589) was diluted 1:200 as per the manufacturer's protocol in Tris-EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH 7.5). SYBR Green I stock solution (Molecular Probes, Eugene, OR, catalog number S7563; InVitrogen Corporation, Carlsbad, CA, catalog number LC6050; Amresco, Solon, OH, catalog number J976) was diluted 1:5000 in TE buffer. SYBR Green I from Molecular Probes was used when not explicitly specified.

DNA standards: Stock solutions of calf thymus DNA (10 mg/ml; Sigma, St. Louis, MO; catalog number D 1501) were prepared in TE or TNE buffer. Secondary stock solutions were prepared by serial dilutions.

Experimental protocol: Equal volumes (250 µl) of a secondary DNA stock solution and the appropriately diluted fluorophore were mixed and transferred to a disposable polystyrene 1.5 ml cuvette that had a 10 mm light path for excitation and 4 mm path for emission (Fisher Scientific, Pittsburgh, PA; catalog number 14-385-942). The mixture was incubated for 5 min at room temperature in the dark before fluorescence measurements were made.

Fluorescence measurements: Fluorescence measurements (photons per second) were made at ambient room temperature, 23-24 °C, using a Spex Fluorolog FL2T2 photon counting spectrofluorometer (Jobin Yvon Inc., Edison, NJ). The excitation monochromators were adjusted to a band width of 20 nm. The emission monochromators were adjusted to a 10 nm band width. These band widths were the same for all four fluorophores. The fluorescence was measured using the excitation and emission wavelengths listed in Table 1.

Precision of the assays: Intra-assay repeatability was evaluated from the replicates of the fluorescence measurements. Inter-assay repeatability was measured using different sources of SYBR Green I (four lots from three suppliers).

Stability of fluorescence: The loss of fluorescence was measured over 24 h using 100 ng/ml and 1000 ng/ml of calf

Table 1. Fluorophore excitation and emission wavelengths

Fluorophore	Excitation	Emission
Ethidium bromide	546 nm	590 nm
Hoechst 33258	346 nm	460 nm
PicoGreen	480 nm	520 nm
SYBR Green I	480 nm	520 nm

These wavelengths were taken from the manufacturer package inserts. The wavelengths were verified as the appropriate optima in the literature and in our own wavelength scans.

Table 2. Comparison of the regression models of the different fluorophores

A: High concentration of DNA (0-20 µg/ml)

Flurophore	p value	R-sq	RMSE	β1	θ
Ethidium Bromide	0.0001	0.9937	35.48	0.06332	560.4
Hoechst 33258	0.0001	0.9925	1789.	5.940	301.2

B: Low concentration of DNA (0-2 ng/ml)

Flurophore	p value	R-sq	RMSE	β1	θ
Ethidium Bromide	0.3000	0.0153	2.608	0.4776	5.462
Hoechst 33258	0.0998	0.0382	557.5	163.0	3.419
PicoGreen	0.0001	0.9737	8.032	71.66	0.1121
SYBR Green I	0.0001	0.9623	13.47	99.86	0.1349

The models of ethidium bromide and Hoechst 33258 with higher concentrations of DNA were significant and had large R^2 values indicating that they are appropriate for measuring DNA in concentrations of 0-20 µg/ml. The same flurophores were unsuitable for measuring lower concentrations of DNA (0-2 ng/ml). The models for PicoGreen and SYBR Green I also suggested that they are appropriate for measuring DNA in concentrations of 0-20 µg/ml. R-sq (R^2) is the fraction of variability explained by the model, RMSE is the root mean square error (in counts/s), β1 (β_1) is the estimated regression coefficient for the fluorescence intensity, and θ is the relative resolution. The values of θ may be compared between experiments of identical design.

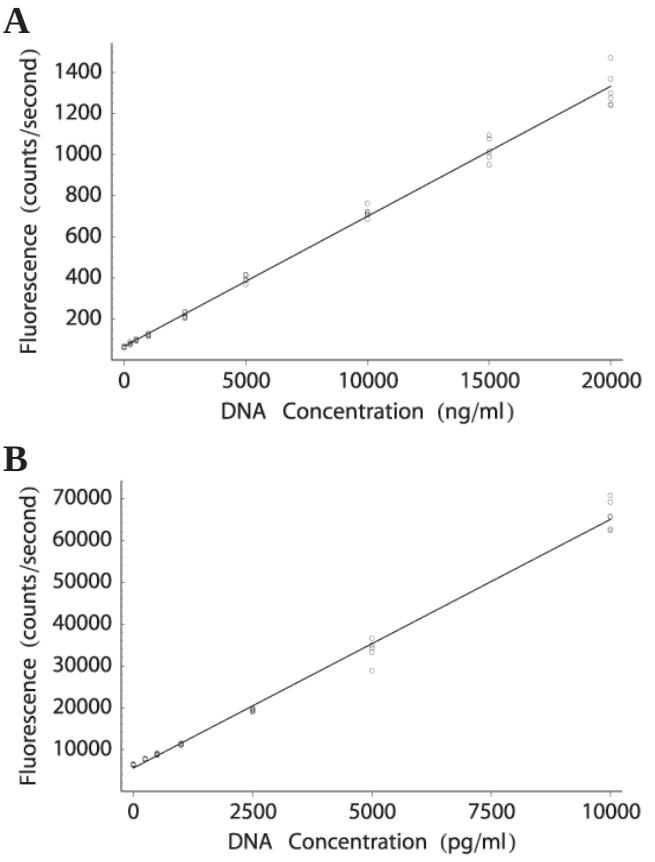

Figure 1. Measurement of high concentrations of DNA (0-20 µg/ml). The graphs show the observed data with the linear regression models for the relationship between DNA concentration and observed fluorescence enhancement. Statistical analysis confirms the validity of each model and suggests that ethidium bromide can distinguish smaller differences in DNA concentration than Hoechst 33258 in this range (Table 2A). **A**: Ethidium bromide. **B**: Hoechst 33258.

thymus DNA in triplicate. The assay was performed using SYBR Green I obtained from three sources (InVitrogen, Amresco, Molecular Probes). The decay rate was determined by fitting the data to an exponential decay model.

Statistical analysis: For each fluorophore, the fluorescence intensity was modeled as a function of concentration of DNA using linear regression (Mathematica version 4.0.1.0, Wolfram Research, Inc., Champaign, IL). A relative scale for comparing the resolution of different calibration curves was used to assess the sensitivity of the different fluorophores. The derivation of this quantity, theta (θ), is explained in Appendix 1. Uniform variance over the modeled range is assumed in the computation of θ.

RESULTS

Quantitation of DNA: In order to evaluate the limits of the linear range of the different fluorophores, DNA samples ranging from pg/ml to μg/ml concentrations were used. The regression models for the four fluorophores mixed with different DNA concentrations are summarized in Table 2. Figure 1 shows that assays of high concentrations of dsDNA using ethidium bromide and Hoechst 33258 are linear over a range extending from 250 ng/ml to 20 μg/ml. These models for ethidium bromide and Hoechst 33258 are statistically significant and have large values of R^2 (Table 2A) indicating that the model accounts for most of the variation seen. At DNA concentrations below 2 ng/ml, the statistical models for the same fluorophores were not significant (Table 2B); this is readily seen in Figure 2A,B. These fluorophores are not usable to measure lower DNA concentrations.

Figure 2C,D show the fluorescence versus DNA concentrations of PicoGreen and SYBR Green I, respectively. The models for PicoGreen and SYBR Green I are statistically significant and have large values of R^2 (Table 2B). The values of theta (θ) show that the calibration curve for PicoGreen has a resolution about 15% smaller than SYBR Green I.

Intra-assay variability: For assays of low concentrations of dsDNA, the coefficients of variation for ethidium bromide, Hoechst 33258, PicoGreen, and SYBR Green I were 7.9%, 8.2%, 8.3%, and 11%, respectively.

Inter-assay variability: Figure 3 shows the plot of fluorescence and DNA concentration from 0-2 μg/ml. The fluo-

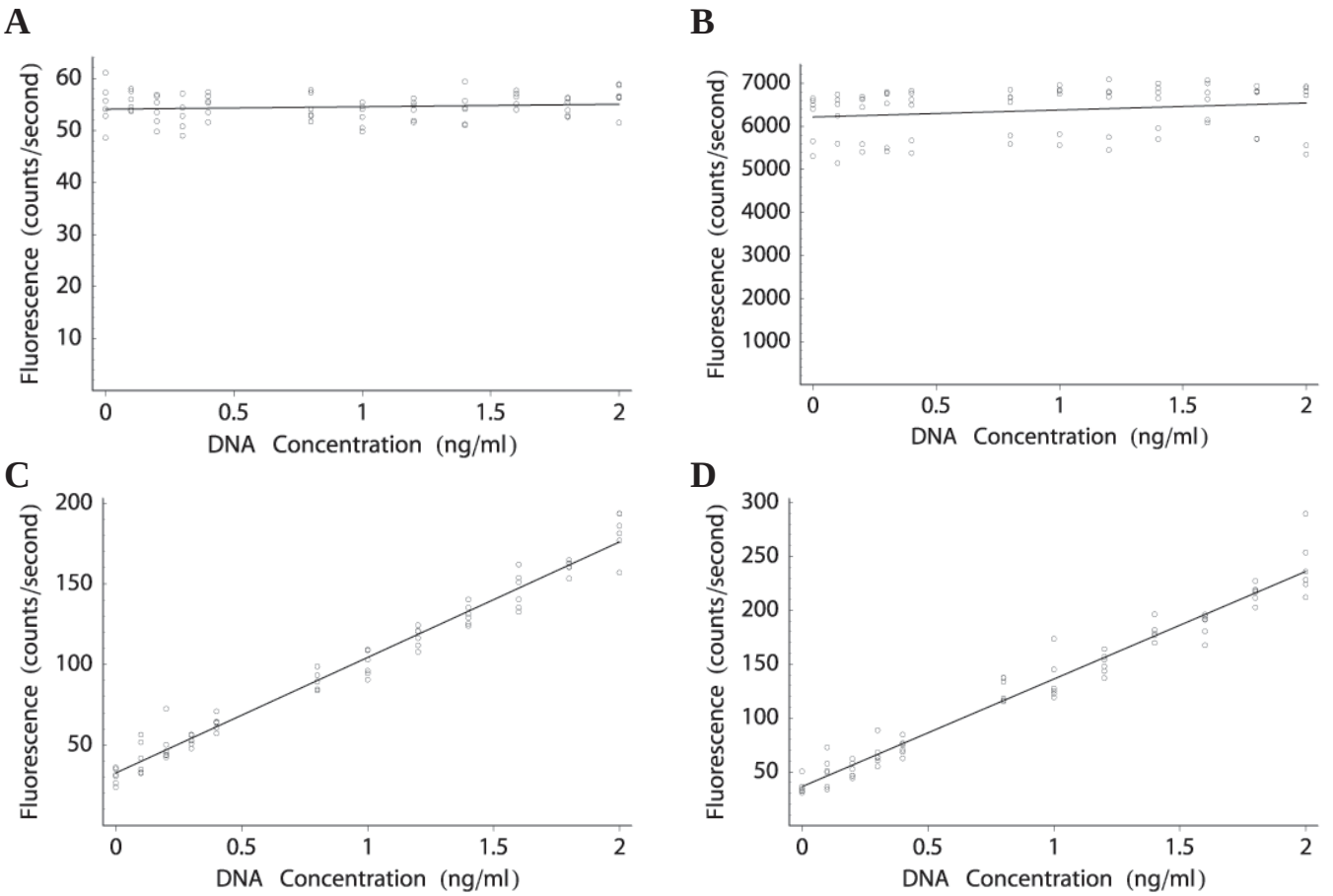

Figure 2. Measurement of low concentrations of DNA (0-2 ng/ml). The graphs show the observed data with the linear regression models for the relationship between DNA concentration and observed fluorescence enhancement. Statistical analysis confirms the validity of the models for PicoGreen and SYBR Green I and suggests that PicoGreen can distinguish smaller differences in DNA concentration in this range (Table 2B). The models for ethidium bromide and Hoechst 33258 are not valid in this range. **A**: Ethidium bromide. **B**: Hoechst 33258. **C**: PicoGreen. **D**: SYBR Green I.

rescence counts are averages of triplicate samples. The coefficients of variation for the assay with SYBR Green I from different sources ranged from 4.6% to 11%.

Stability of Fluorescence: The stability of the complexes of SYBR Green I and dsDNA is illustrated in Figure 4. The fluorescence emitted by the SYBR Green I bound dsDNA (100 ng/ml and 1000 ng/ml) was measured over 24 h. The relative fluorescence was calculated as a ratio of the fluorescence emitted at time t to the fluorescence emitted at the start of the experiment. A systematic degradation of fluorescence was observed. The decay constant was 2.5% for the 100 ng/ml concentration of DNA and 0.9% for the 1000 ng/ml of DNA based on an exponential model of decay. With a higher concentration of DNA, a lower rate of decay was observed.

DISCUSSION

In vision research, a variety of factors can limit the amount of tissue available. Species like mouse, zebrafish, and *Drosophila* have eyes that are quite small. Experiments using regional or sectoral information from a single eye of larger species also require the use of modest tissue samples. The number of cells obtained from ocular tumors or using microdissection techniques (e.g., laser capture microscopy) is similarly small. In polymorphism analyses, many tests may need to be performed on a single sample of genomic DNA. Additionally, any time reagents are expensive, using small amounts of tissue allows for the analysis of large numbers of experimental samples. For these reasons, the ability to work with small quantities of DNA is useful and often necessary in vision research.

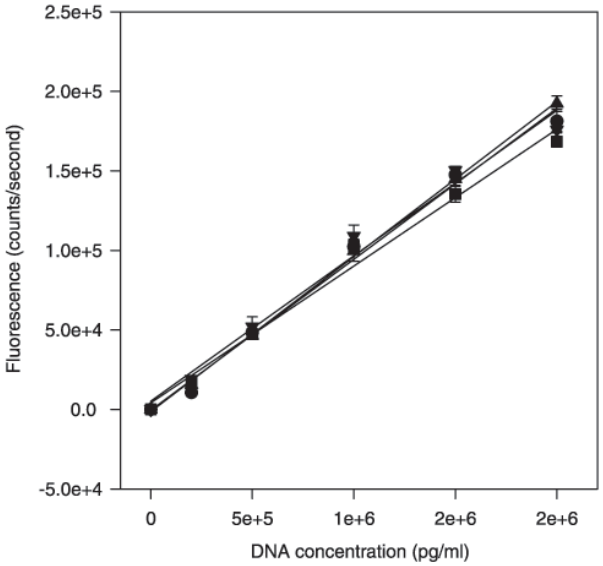

Figure 3. Inter-assay variability. The repeatability of the DNA fluorescence assay was tested using SYBR Green I (four lots from three suppliers). The graphs show the means of the observed data with error bars and the linear regression models for the relationship between DNA concentration and observed fluorescence enhancement for each lot. The coefficients of variation for the assay ranged from 4.6% to 11%.

In choosing a method of measuring dsDNA, one must consider several factors: sensitivity, discrimination, special requirements of the investigation, and cost. The traditional method for determining the concentration of DNA in solution is by measuring absorbance at 260 nm. The A_{260} method is accurate and reproducible if the samples are adequately purified and an adequate amount of material is available. However, this measurement is relatively insensitive with a practical limit of detection of about 150 ng/ml (assuming that the standard deviation of absorbance measurements at 260 nm is 0.001) [7]. If samples are not purified, the presence of RNA or protein in the samples could result in overestimation of the DNA concentration. Also, absorbance measurements at 260 nm require expensive quartz cuvettes that require careful cleaning and maintenance.

From our data, the relative resolutions of ethidium bromide, Hoechst 33258, PicoGreen, and SYBR Green I were 560.4, 301.2, 0.1121, and 0.1349, respectively. Thus, PicoGreen and SYBR Green I were more than one thousand times more sensitive than ethidium bromide and Hoechst 33528.

The SYBR Green I and PicoGreen fluorophores are particularly convenient and practical because their excitation frequencies are in the visible spectrum. On a cost per assay basis, SYBR Green I (US$0.0058/assay) is substantially less expensive than PicoGreen (US$0.19/assay). An additional cost benefit of fluorescence assays (rather than absorbance measurements) is that they use inexpensive, disposable cuvettes.

This study has shown that assays using SYBR Green I and PicoGreen are sufficiently sensitive to measure low concentrations (0-2 ng/ml) of dsDNA. Ethidium bromide and Hoechst 33258 are only appropriate for higher concentrations (0-20 μg/ml) of DNA.

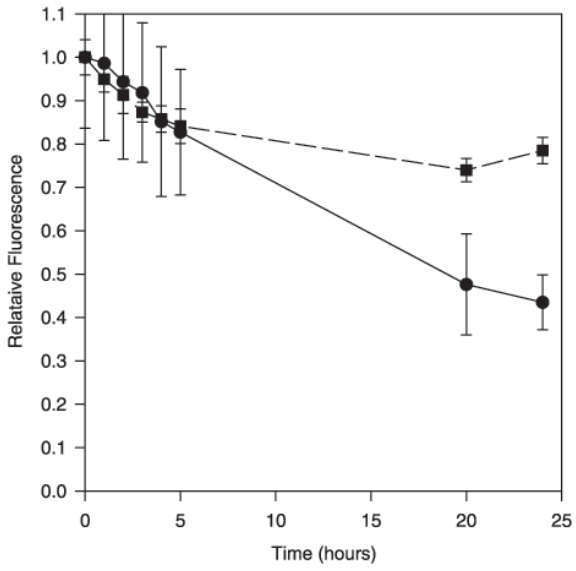

Figure 4. Stability of fluorescence. The fluorescence enhancement of the SYBR Green I-dsDNA complex was measured over a 24 h period to assess the stability of the complex. DNA concentrations of 100 ng/ml (solid line) and 1000 ng/ml (dashed line) are shown.

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Appendix 1. Estimating sensitivity

This appendix explains the method used to assess the resolutions of the fluorophore systems. The data were collected by measuring the fluorescence enhancement of solutions of known concentrations of dsDNA. Linear models of the expected value of Y given X (E(Y|X)) were developed of the form:

$$E(Y|X) = \beta_1 X + \beta_0 \qquad \text{(Eq. 1)}$$

The resolution for each method is the smallest difference that can be detected by that method. We chose a statistical criteria for detecting the differences. To do this, we used a balanced design that greatly simplifies the computations. In computations, we also assumed the usual regression assumption of homoscedasticity held, though this was verified after the data were collected. Our approach used the t-test for detecting significant differences. It can be shown that for a two sample t-test [9] with a balanced design and equal variance (s_e^2), the test statistic is:

$$t = \frac{Y_1 - Y_2}{s_e\sqrt{2/n}} \qquad \text{(Eq. 2)}$$

Substituting equation 1 in equation 2 and simplifying, we see that:

$$t = \frac{\beta_1(X_1 - X_2)}{s_e\sqrt{2/n}} \qquad \text{(Eq. 3)}$$

At the point where statistical significance is reached, the test statistic equals the critical value (t_c) and we can solve for resolution (ΔX):

$$\Delta X = \sqrt{\frac{2}{n}} t_c \frac{s_e}{\beta_1} \qquad \text{(Eq. 4)}$$

Within an experiment, t_c and n are constant. These are also constant across experiments of identical design. Estimates of the other quantities are easily taken from the output of regression software. The slope of the regression line estimates Œ≤1 and the mean square error estimates s_e^2 (or root mean square error estimates s_e). This allows us to easily compare the resolutions from identically designed experiments using the relative resolutions (θ):

$$\Delta X \propto \frac{s_e}{\beta_1} = \theta \qquad \text{(Eq. 5)}$$

Thus, large values of β_1 give a better resolution, as we would expect. Similarly, the statistical noise seen in a larger variance limits the resolution.

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