

Quantitation of DNA and RNA with Absorption and Fluorescence Spectroscopy

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APPENDIX 3D

ABSTRACT

Quantitation of nucleic acids is a fundamental tool in molecular biology that requires accuracy, reliability, and the use of increasingly smaller sample volumes. This unit describes the traditional absorbance measurement at 260 nm and three more sensitive fluorescence techniques employing Hoechst 33258, ethidium bromide, and PicoGreen. The range of the assays covers 25 pg/ml to 50 µg/ml. Absorbance at 260 nm has an effective range from 1 to 50 µg/ml; Hoechst 33258 from 0.01 to 15 µg/ml; ethidium bromide from 0.1 to 10 µg/ml; and PicoGreen from 25 to 1000 pg/ml. *Curr. Protoc. Mol. Biol.* 93:A.3D.1-A.3D.14. © 2011 by John Wiley & Sons, Inc.

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INTRODUCTION

Reliable quantitation of picogram, nanogram, and microgram amounts of DNA and RNA in solution is essential to researchers in molecular biology. In addition to absorbance measurements at 260 nm (see Basic Protocol), more sensitive fluorescence techniques are presented that employ Hoechst 33258 (see Alternate Protocol 1), ethidium bromide (see Alternate Protocol 2), and PicoGreen (see Alternate Protocol 3).

Absorbance measurements are straightforward as long as any contributions from contaminants and the buffer components are taken into account. Fluorescence assays are less prone to interference than A_{260} measurements. Fluorophore excitation and subsequent emission produce higher sensitivity, and many fluorophores provide specificity by preferentially binding to the sample material of interest (e.g., dsDNA). Three classes of fluorophores are commonly used for nucleic acid detection: intercalating dyes such as ethidium bromide, fluorophores that bind to the minor groove of dsDNA such as Hoechst dye, and other specialized fluorescent stains such as cyanine dyes. Cyanine dyes commonly used for nucleic acid detection include PicoGreen for dsDNA, OliGreen for oligonucleotides, and RiboGreen for RNA. Although fluorescent assays require more steps, they are simple to perform. As with absorbance measurements, a reading from the reagent blank is taken prior to adding the DNA. In instruments where the readout can be set to indicate concentration, a known concentration is used for calibration and subsequent readings are taken in µg/ml, ng/ml, or pg/ml DNA.

Biomolecular assays are continually being developed that require progressively smaller amounts of nucleic acids for analysis. Several novel microvolume quantitation systems have been developed in recent years. These systems not only greatly reduce the amount of sample required for analysis (~1 µl), but also employ much shorter path lengths that greatly expand the dynamic range of possible concentrations, essentially removing the need to perform dilutions. The efficiency and ease of use of microvolume methodologies has made them widely accepted alternatives to traditional nucleic acid quantitation

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methods, even when the sample is plentiful. A full discussion of microvolume technologies and the associated nucleic acid quantitation protocols may be found in *APPENDIX 3J* (Microvolume Quantitation of Nucleic Acids).

DETECTION OF NUCLEIC ACIDS USING ABSORPTION SPECTROSCOPY

Absorption of the sample is measured at several different wavelengths to assess purity and concentration of nucleic acids. A_{260} measurements are quantitative for relatively pure nucleic acid preparations in microgram quantities. Absorbance readings cannot discriminate between DNA and RNA; however, the ratio of A at 260 and 280 nm can be used as an indicator of nucleic acid purity. Proteins, for example, have a peak absorption at 280 nm that will reduce the A_{260}/A_{280} ratio. Absorbance at 325 nm indicates particulates in the solution or dirty cuvettes; contaminants containing peptide bonds or aromatic moieties such as protein and phenol absorb at 230 nm.

This protocol is designed for a single-beam ultraviolet to visible range (UV-vis) spectrophotometer. If available, a double-beam spectrophotometer will simplify the measurements, as it will automatically compare the cuvette holding the sample solution to a reference cuvette that contains the blank. In addition, more sophisticated double-beam instruments will scan various wavelengths and report the results automatically.

Materials

- 1 × TNE buffer (see recipe)
- DNA sample to be quantitated
- Calf thymus DNA standard solutions (see recipe)
- Matched quartz semi-micro spectrophotometer cuvettes (1-cm pathlength)
- Single- or dual-beam spectrophotometer (ultraviolet to visible)

1. Pipet 1.0 ml of 1 × TNE buffer into a quartz cuvette. Place the cuvette in a single- or dual-beam spectrophotometer, read at 325 nm (note contribution of the blank relative to distilled water if necessary), and zero the instrument. Use this blank solution as the reference in double-beam instruments. For single-beam spectrophotometers, remove blank cuvette and insert cuvette containing DNA sample or standard suspended in the same solution as the blank. Take reading. Repeat this process at 280, 260, and 230 nm.

It is important that the DNA be suspended in the same solution as the blank.

2. To determine the concentration (C) of DNA present, use the A_{260} reading in conjunction with one of the following equations:

$$\text{Single-stranded DNA: } C (\text{pmol}/\mu\text{l}) = \frac{A_{260}}{10 \times S}$$

$$C (\mu\text{g}/\text{ml}) = \frac{A_{260}}{0.027}$$

$$\text{Double-stranded DNA: } C (\text{pmol}/\mu\text{l}) = \frac{A_{260}}{13.2 \times S}$$

$$C (\mu\text{g}/\text{ml}) = \frac{A_{260}}{0.020}$$

$$\text{Single-stranded RNA: } C (\mu\text{g}/\text{ml}) = \frac{A_{260}}{0.025}$$

$$\text{Oligonucleotide: } C (\text{pmol}/\mu\text{l}) = A_{260} \times \frac{100}{1.5 N_A + 0.71 N_C + 1.20 N_G + 0.84 N_T}$$

Table A.3D.1 Molar Extinction Coefficients of DNA Bases^a

Base	$\epsilon_{260\text{ nm}}^{1\text{M}}$
Adenine	15,200
Cytosine	7,050
Guanine	12,010
Thymine	8,400

^aMeasured at 260 nm; see Wallace and Miyada (1987). Detailed spectrophotometric properties of nucleoside triphosphates are listed in *UNIT 3.4*.

Table A.3D.2 Spectrophotometric Measurements of Purified DNA^a

Wavelength (nm)	Absorbance	A_{260}/A_{280}	Conc. ($\mu\text{g/ml}$)
325	0.01	—	—
280	0.28	—	—
260	0.56	2.0	28
230	0.30	—	—

^aTypical absorbancy readings of highly purified calf thymus DNA suspended in $1 \times$ TNE buffer. The concentration of DNA was nominally 25 $\mu\text{g/ml}$.

where S represents the size of the DNA in kilobases and N is the number or residues of base A, G, C, or T.

For double- or single-stranded DNA and single-stranded RNA: These equations assume a 1-cm-pathlength spectrophotometer cuvette and neutral pH. The calculations are based on the Lambert-Beer law, $A = \epsilon Cl$, where A is the absorbance at a particular wavelength, C is the concentration of DNA, l is the pathlength of the spectrophotometer cuvette (typically 1 cm), and ϵ is the extinction coefficient. For solution concentrations given in mol/liter (M) and a cuvette of 1-cm pathlength, ϵ is the molar extinction coefficient and has units of $M^{-1}\text{cm}^{-1}$. If concentration units of $\mu\text{g/ml}$ are used, then ϵ is the specific absorption coefficient and has units of $(\mu\text{g/ml})^{-1}\text{cm}^{-1}$. The values of ϵ used here are as follows: ssDNA, $0.027 (\mu\text{g/ml})^{-1}\text{cm}^{-1}$; dsDNA, $0.020 (\mu\text{g/ml})^{-1}\text{cm}^{-1}$; ssRNA, $0.025 (\mu\text{g/ml})^{-1}\text{cm}^{-1}$. Using these calculations, an A_{260} of 1.0 indicates 50 $\mu\text{g/ml}$ double-stranded DNA, $\sim 37 \mu\text{g/ml}$ single-stranded DNA, or $\sim 40 \mu\text{g/ml}$ single-stranded RNA (adapted from Applied Biosystems, 1987).

For oligonucleotides: Concentrations are calculated in the more convenient units of $\text{pmol}/\mu\text{l}$. The base composition of the oligonucleotide has significant effects on absorbance, because the total absorbance is the sum of the individual contributions of each base (Table A.3D.1).

3. Use the A_{260}/A_{280} ratio and readings at A_{230} and A_{325} to estimate the purity of the nucleic acid sample.

Ratios of 1.8 to 1.9 and 1.9 to 2.0 indicate highly purified preparations of DNA and RNA, respectively. Contaminants that absorb at 280 nm (e.g., protein) will lower this ratio.

Absorbance at 230 nm reflects contamination of the sample by phenol or urea, whereas absorbance at 325 nm suggests contamination by particulates and dirty cuvettes. Light scatter at 325 nm can be magnified 5-fold at 260 nm (K. Hardy, pers. comm.).

Typical values at the four wavelengths for a highly purified preparation are shown in Table A.3D.2.

DNA DETECTION USING THE DNA-BINDING FLUOROCHROME HOECHST 33258

Use of fluorometry to measure DNA concentration has gained popularity because it is simple and much more sensitive than spectrophotometric measurements. Specific for nanogram amounts of DNA, the Hoechst 33258 fluorochrome has little affinity for RNA and works equally well with either whole-cell homogenates or purified preparations of DNA. The fluorochrome is, however, sensitive to changes in DNA composition, with preferential binding to AT-rich regions. A fluorometer capable of an excitation wavelength of 365 nm and an emission wavelength of 460 nm is required for this assay.

Additional Materials (also see Basic Protocol)

Hoechst 33258 assay solution (working solution; see recipe)
Dedicated filter fluorometer (Hoefer DQ 300, Promega QuantiFluor, or Invitrogen Qubit) *or* scanning fluorescence spectrophotometer (Shimadzu RF-5301PC, Hitachi F-2500, or Horiba FluoroMax, <http://www.horiba.com>)
Fluorometric square glass cuvettes *or* disposable acrylic cuvettes (Sarstedt)
Calf thymus DNA standard solution (see recipe)
Teflon stir rod appropriate for cuvettes

NOTE: The DNAQF Kit (Sigma-Aldrich) contains the Hoechst 33258, buffers, and DNA standards for the assay.

1. Prepare the scanning fluorescence spectrophotometer by setting the excitation wavelength to 365 nm and the emission wavelength to 460 nm.

The dedicated filter fluorometer has fixed wavelengths at 365 and 460 nm and does not need adjustment.

2. Pipet 2.0 ml Hoechst 33258 assay solution into cuvette and place in sample chamber. Take a reading without DNA and use this as the background measurement.

If the fluorometer has a concentration readout mode or is capable of creating a standard curve, set instrument to read 0 with the blank solution. Otherwise, note the readings in relative fluorescence units. Be sure to take a blank reading for each cuvette used, as slight variations can cause changes in the background reading.

3. With the cuvette still in the sample chamber, add 2 μ l of a DNA standard to the blank Hoechst 33258 assay solution. Mix in the cuvette with a Teflon stir rod or by capping and inverting the cuvette. Read emission in relative fluorescence units or set the concentration readout equal to the final DNA concentration. Repeat measurements with remaining DNA standards using fresh assay solution (record background zero reading and zero instrument if needed).

If necessary, the DNA standards should be quantitated by A_{260} measurement (Basic Protocol) before being used here.

Small-bore tips designed for loading sequencing gels minimize errors of pipetting small volumes. Prerinse tips with sample and make sure no liquid remains outside the tip after drawing up the sample.

Read samples in duplicate or triplicate, with a blank reading taken each time. Unusual or unstable blank readings indicate a dirty cuvette or particulate material in the solution, respectively.

4. Repeat step 3 with unknown samples.

A dye concentration of 0.1 μ g/ml is adequate for final DNA concentrations up to \sim 500 ng/ml. Increasing the working dye concentration to 1 μ g/ml Hoechst 33258 will extend the assay's range to 15 μ g/ml DNA, but will limit sensitivity at low concentrations (5 to 10 ng/ml). Sample volumes of \leq 10 μ l can be added to the 2.0-ml aliquot of Hoechst 33258 assay solution.

DNA AND RNA DETECTION WITH ETHIDIUM BROMIDE FLUORESCENCE

ALTERNATE PROTOCOL 2

In contrast to the fluorochrome Hoechst 33258, ethidium bromide fluorescence is relatively unaffected by differences in the base composition of DNA. Ethidium bromide is not as sensitive as Hoechst 33258 and, although capable of detecting nanogram levels of DNA, will also bind to RNA. In preparations of DNA with minimal RNA contamination or with DNA samples having an unusually high guanine and cytosine (GC) content where the Hoechst 33258 signal can be quite low, ethidium bromide offers a relatively sensitive alternative to the more popular Hoechst 33258 DNA assay. A fluorometer capable of an excitation wavelength of 302 or 546 nm and an emission wavelength of 590 nm is required for this assay.

Additional Materials (also see Basic Protocol)

Ethidium bromide assay solution (see recipe)

1. Pipet 2.0 ml ethidium bromide assay solution into cuvette and place in sample chamber. Set excitation wavelength to 302 nm or 546 nm and emission wavelength to 590 nm. Take an emission reading without DNA and use as background.

If the instrument has a concentration readout mode or is capable of creating a standard curve, set instrument to read 0 with the blank solution. Otherwise note the readings in relative fluorescence units.

The excitation wavelength of this assay can be either in the UV range (~302 nm) using a quartz cuvette or in the visible range (546 nm) using a glass cuvette. In both cases the emission wavelength is 590 nm.

2. Read and calibrate these samples as described in step 3 of the Hoechst 33258 assay (Alternate Protocol 1).
3. Read emissions of the unknown samples as in step 4 of the Hoechst 33258 assay.

A dye concentration of 5 $\mu\text{g/ml}$ in the ethidium bromide assay solution is appropriate for final DNA concentrations up to 1000 ng/ml. 10 $\mu\text{g/ml}$ ethidium bromide in the ethidium bromide assay solution will extend the assay's range to 10 $\mu\text{g/ml}$ DNA, but is only used for DNA concentrations >1 $\mu\text{g/ml}$. Sample volumes of up to 10 μl can be added to the 2.0-ml aliquot of ethidium bromide assay solution.

DNA DETECTION USING PICOGREEN dsDNA QUANTITATION REAGENT

ALTERNATE PROTOCOL 3

PicoGreen dsDNA quantitation reagent enables quantitation of as little as 25 pg/ml of dsDNA (50 pg dsDNA in a 2-ml assay volume) with a standard spectrofluorometer and fluorescein-excitation and emission wavelengths. This sensitivity exceeds that achieved with the Hoechst 33258-based assay (Alternate Protocol 1) by 400-fold. Using a fluorescence microplate reader, it is possible to detect as little as 250 pg/ml dsDNA (50 pg in a 200- μl assay volume). The standard PicoGreen assay protocol is also simpler than that for Hoechst 33258 because a single concentration of the PicoGreen reagent allows detection over the full dynamic range of the assay. In order to achieve more than two orders of magnitude in dynamic range with Hoechst-based assays, two different dye concentrations are recommended. In contrast, the linear detection range of the PicoGreen assay in a standard fluorometer extends over more than four orders of magnitude in DNA concentration—from 25 pg/ml to 1000 ng/ml—with a single dye concentration (Fig. A.3D.1). Linearity is maintained in the presence of several compounds that commonly contaminate nucleic acid preparations, including salts, urea, ethanol, chloroform, detergents, proteins, and agarose.

CAUTION: No data are available addressing the mutagenicity or toxicity of PicoGreen dsDNA quantitation reagent. Because this reagent binds to nucleic acids, it should be

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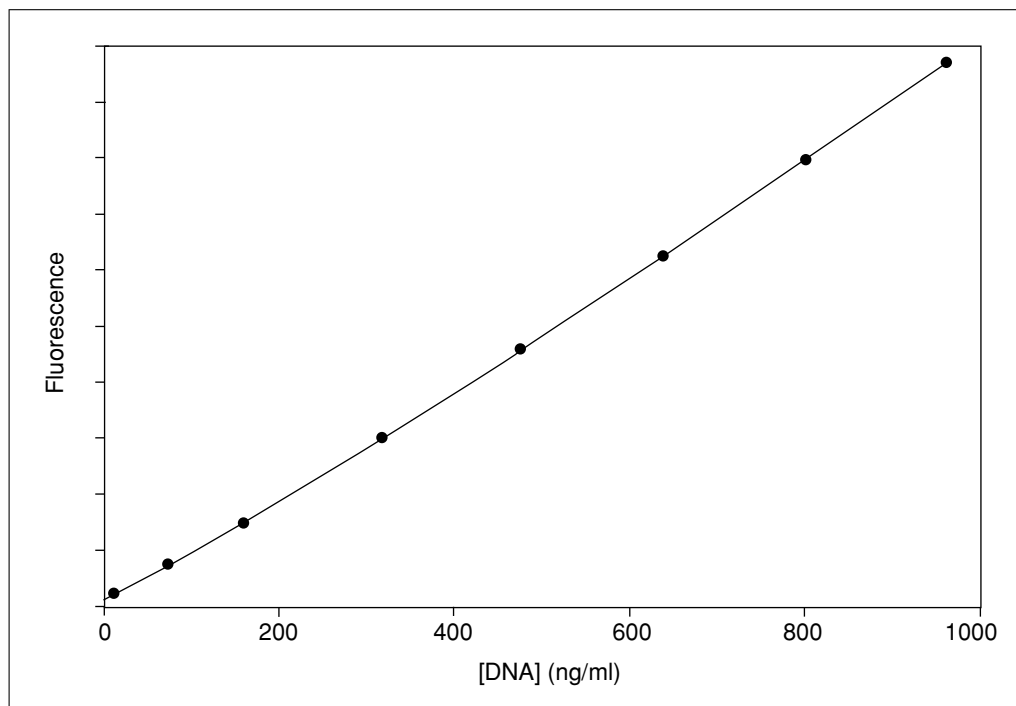


Figure A.3D.1 Dynamic range and sensitivity of the PicoGreen dsDNA quantitation assay. Calf thymus DNA was added to cuvettes containing PicoGreen dsDNA quantitation reagent diluted in 10 mM Tris-Cl/1 mM EDTA, pH 7.5 (TE). The samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm using a spectrofluorometer. Fluorescence emission intensity was then plotted versus DNA concentration.

treated as a potential mutagen and handled with appropriate care. The DMSO stock solution should be handled with particular caution, as DMSO is known to facilitate the entry of organic molecules into tissues. It is strongly recommended that double gloves be used when handling the DMSO stock solution. As with all nucleic acid reagents, solutions of PicoGreen reagent should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dye.

Additional Materials (also see *Basic Protocol*)

PicoGreen dsDNA quantitation kit (Invitrogen) containing:

PicoGreen dsDNA quantitation reagent (Component A), 1 ml solution in DMSO (store frozen up to 6 months at -20°C , protected from light)

20 \times TE (Component B), 25 ml of 200 mM Tris-Cl/20 mM EDTA, pH 7.5 (store up to 6 months at 4°C ; may be frozen for long-term storage)

Lambda DNA standard (Component C), 1 ml of 100 $\mu\text{g/ml}$ in TE (store up to 6 months at 4°C ; may be frozen for long-term storage)

Spectrofluorometer or fluorescence microplate reader

NOTE: For either the kits or the stand-alone reagent, sufficient reagent is supplied for 200 assays using an assay volume of 2 ml according to the protocol below. Note that the assay volume is dependent on the instrument used to measure fluorescence; with a microplate reader and a 96-well microplate, the assay volume is reduced to 200 μl and 2000 assays are possible. The PicoGreen reagent supplied in the kits is exactly the same as the reagent sold separately. The DMSO stock solution should be stored frozen at -20°C and protected from light. The 20 \times assay buffer and lambda DNA standard in the kits are best stored at 4°C ; however, either may be frozen for long-term storage. When properly stored, components should be stable for at least 6 months.

Prepare reagent

1. On day of experiment, prepare an aqueous working solution of the PicoGreen reagent by making a 200-fold dilution of the concentrated DMSO solution in $1 \times$ TE.

Allow the PicoGreen reagent to warm to room temperature before opening the vial. Because the PicoGreen dye is an extremely sensitive detection reagent for dsDNA, it is imperative that the TE solution be free of contaminating nucleic acids. The $20 \times$ TE buffer included in the PicoGreen dsDNA Quantitation Kits is certified to be nucleic acid-free and DNase-free. Prepare a $1 \times$ TE working solution by diluting the concentrated buffer 20-fold with sterile, distilled, DNase-free water.

To prepare sufficient working solution to assay 20 samples in a 2-ml final volume, add 100 μ l PicoGreen dsDNA quantitation reagent to 19.9 ml TE. The solution should be prepared in a plastic container rather than glass, as the reagent may adsorb to glass surfaces. Protect the working solution from light by covering it with foil or placing it in the dark, as the PicoGreen reagent is susceptible to photodegradation. For best results, this solution should be used within a few hours of preparation.

Establish DNA standard curve

2. Prepare a 2 μ g/ml stock solution of dsDNA in $1 \times$ TE. Determine the DNA concentration on the basis of absorbance at 260 nm (A_{260}) in a cuvette with a 1-cm pathlength (see Basic Protocol); an A_{260} of 0.04 corresponds to 2 μ g/ml dsDNA solution.

For a standard curve, the author commonly uses bacteriophage lambda DNA (provided with kit) or calf thymus DNA, although any purified dsDNA preparation may be used. The lambda DNA standard, provided at 100 μ g/ml in the PicoGreen kits, can simply be diluted 50-fold in $1 \times$ TE to make the 2 μ g/ml working solution. For example, 30 μ l of the DNA standard mixed with 1.47 ml of TE will be sufficient for the standard curve described below. It is sometimes preferable to prepare the standard curve with DNA similar to the type being assayed; e.g., long or short linear DNA fragments when quantitating similar-sized restriction fragments or plasmid when quantitating plasmid DNA. However, most linear dsDNA molecules yield approximately equivalent signals, regardless of fragment length. Results have shown that the PicoGreen assay remains linear in the presence of several compounds that commonly contaminate nucleic acid preparations, although the signal intensity may be affected (Table A.3D.3). Thus, to serve as an effective control, the dsDNA solution used to prepare the standard curve should be treated the same way as the experimental samples and should contain similar levels of such compounds.

- 3a. *For high-range standard curve:* Create a five-point standard curve from 1 ng/ml to 1 μ g/ml by combining the 2 μ g/ml stock prepared in step 12 with $1 \times$ TE, in disposable cuvettes (or plastic test tubes for transfer to quartz cuvettes), according to Table A.3D.4.
- 3b. *For low-range standard curve:* Prepare a 40-fold dilution of the 2 μ g/ml DNA solution to yield a 50 ng/ml DNA stock solution. Create a five-point standard curve from 25 pg/ml to 25 ng/ml by combining this 50 ng/ml stock with $1 \times$ TE in disposable cuvettes (or plastic test tubes for transfer to quartz cuvettes), according to Table A.3D.4.

To create the low-range standard curve, adjust the fluorometer gain to accommodate the lower fluorescence signals.

4. Add 1.0 ml of the aqueous working solution of PicoGreen reagent (prepared in step 1) to each cuvette. Mix well and incubate for 2 to 5 min at room temperature, protected from light.
5. After incubation, measure sample fluorescence using a spectrofluorometer or fluorescence microplate reader and standard fluorescein wavelengths (excitation \sim 480 nm, emission \sim 520 nm). To ensure that the sample readings remain in the detection range

Table A.3D.3 Effects of Common Nucleic Acid Contaminants on Signal Intensity in the PicoGreen dsDNA Quantitation Assay

Compound	Concentration	% signal change ^a
<i>Salts</i>		
Ammonium acetate	50 mM	3% decrease
Sodium acetate	30 mM	3% increase
Sodium chloride	200 mM	30% decrease
Zinc chloride	5 mM	8% decrease
Magnesium chloride	50 mM	33% decrease
Urea	2 M	9% increase
<i>Organic solvents</i>		
Phenol	0.1%	13% increase
Ethanol	10%	12% increase
Chloroform	2%	14% increase
<i>Detergents</i>		
Sodium dodecyl sulfate	0.01%	1% decrease
Triton X-100	0.1%	7% increase
<i>Proteins</i>		
Bovine serum albumin	2%	16% decrease
IgG	0.1%	19% increase
<i>Other compounds</i>		
Polyethylene glycol	2%	8% increase
Agarose	0.1%	4% increase

^aThe compounds were incubated at the indicated concentrations with PicoGreen reagent in the presence of 500 ng/ml calf thymus DNA. All samples were assayed in a final volume of 200 μ l in 96-well microplates using a CytoFluor microplate reader (PerSeptive Biosystems). Samples were excited at 485 nm and fluorescence intensity was measured at 520 nm.

of the fluorometer, set the instrument's gain so that the sample containing the highest DNA concentration yields a fluorescence intensity near the fluorometer's maximum. To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.

6. Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus DNA concentration (see Fig. A.3D.1).

Analyze samples

7. Add 1.0 ml of the aqueous working solution of the PicoGreen reagent (prepared in step 1) to each sample. Incubate 2 to 5 min at room temperature, protected from light.
8. Measure fluorescence of samples using instrument parameters that correspond to those used when generating standard curve (see steps 2 to 6). To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.
9. Subtract the fluorescence value of the reagent blank from that of each sample. Determine the DNA concentration of the samples from standard curve.
10. If desired, repeat assay using a different dilution of the sample to confirm results.

Table A.3D.4 Preparing Standard Curve with the PicoGreen Reagent

Vol. (μl) DNA stock	Vol. (μl) 1 × TE	Vol. (μl) PicoGreen working solution	Final DNA conc. in PicoGreen assay
<i>High-range (stock contains 2 μg/ml DNA)^a</i>			
1000	0	1000	1 μg/ml
100	900	1000	100 ng/ml
10	990	1000	10 ng/ml
1	999	1000	1 ng/ml
0	1000	1000	Blank
<i>Low range (stock contains 50 ng/ml DNA)^b</i>			
1000	0	1000	25 ng/ml
100	900	1000	2.5 ng/ml
10	990	1000	250 pg/ml
1	999	1000	25 pg/ml
0	1000	1000	Blank

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Calf thymus DNA standard solutions

Kits containing calf thymus DNA standard for fluorometry are available. Premeasured, CsCl-gradient-purified DNA of defined GC content, for use in absorption and fluorometric spectroscopy, is available from Sigma (e.g., calf thymus DNA, 42% GC; cat. no. D4764). Reconstitute DNA stock to a concentration of 1000 μg/ml in TE buffer (APPENDIX 2) and dilute to 500, 250, 100, 50, 25, and 10 μg/ml in TE buffer, to obtain calibration curve (Tables A.3D.5 and A.3D.6).

Ethidium bromide assay solution

Add 10 ml of 10× TNE buffer (see recipe) to 89.5 ml H₂O. Filter through a 0.45-μm filter, then add 0.5 ml of 1 mg/ml ethidium bromide.

Add the dye after filtering, as ethidium bromide will bind to most filtration membranes.

CAUTION: Ethidium bromide is hazardous; wear gloves and use appropriate care in handling, storage, and disposal.

Hoechst 33258 assay solutions

Stock solution: Dissolve in H₂O at 1 mg/ml. Stable for ~6 months at 4°C.

Working solution: Add 10 ml of 10× TNE buffer (see recipe) to 90 ml H₂O. Filter through a 0.45-μm filter, then add 10 μl of 1 mg/ml Hoechst 33258.

Hoechst 33258 (Sigma-Aldrich, cat. no. 14530) is a fluorochrome dye with a molecular weight of 624 and a molar extinction coefficient of $4.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 338 nm.

The dye is added after filtering because it will bind to most filtration membranes.

CAUTION: Hoechst 33258 is hazardous; use appropriate care in handling, storage, and disposal.

Table A.3D.5 Preparing Standard Curve for Hoechst 33258 Assay: Preparing Standards

1000 µg/ml DNA stock volume	Volume TE buffer	DNA standard concentration
500 µl	500 µl	500 µg/ml
250 µl	750 µl	250 µg/ml
100 µl	900 µl	100 µg/ml
50 µl	950 µl	50 µg/ml
25 µl	975 µl	25 µg/ml
10 µl	990 µl	10 µg/ml

Table A.3D.6 Preparing Standard Curve for Hoechst 33258 Assay: The Standard Assay

DNA standard (Table A.3D.5)	Volume standard	Volume Hoechst 33258 assay solution (see recipe)	DNA concentration, final
500 µg/ml	2 µl	2.0 ml	500 ng/ml
250 µg/ml	2 µl	2.0 ml	250 ng/ml
100 µg/ml	2 µl	2.0 ml	100 ng/ml
50 µg/ml	2 µl	2.0 ml	50 ng/ml
25 µg/ml	2 µl	2.0 ml	25 ng/ml
10 µg/ml	2 µl	2.0 ml	10 ng/ml

TNE buffer, 10×

100 mM Tris base

10 mM EDTA

2.0 M NaCl

Adjust pH to 7.4 with concentrated HCl

As needed, dilute with H₂O to desired concentration

COMMENTARY

Background Information

In deciding which method of nucleic acid measurement is appropriate, three primary issues are critical: specificity, sensitivity, and interfering substances. Secondary issues include the potential uncertainty associated with sample dilution, possible reagent preparation, and complexity of the respective instrumentation. Properties of the assays described in this unit are listed in Table A.3D.7. The traditional, most convenient, and most commonly utilized method for determining the amount of DNA in solution is by measuring absorbance at 260 nm. Because many potential contaminants of DNA and RNA preparations also absorb in the UV range, absorption spectroscopy is a reliable method to assess both the purity of a preparation and the quantity of DNA or RNA present. Absorption spectroscopy does

have serious limitations. For conventional 1-cm pathlength spectrophotometers, relatively large amounts of DNA are required to get accurate readings—for example, 500 ng/ml DNA is equivalent to only 0.01 A_{260} units. Although microvolume methods (*APPENDIX 3J*) greatly reduce the sample mass requirement, no spectroscopy method can discriminate between RNA and DNA, and UV-absorbing contaminants such as protein will cause discrepancies.

The fluorescence assay using Hoechst 33258 dye (Alternate Protocol 1) is the first procedure in common use that has specificity for DNA under defined circumstances (i.e., it does not measure RNA). That made it the original method of choice for rapid measurement of small quantities of DNA, with a detection limit of ~1 ng DNA. Concentrations of DNA

Table A.3D.7 Properties of Absorbance and Fluorescence Spectrophotometric Assays for DNA and RNA

Property	Absorbance (A_{260})	Fluorescence		
		H33258	Ethidium bromide	PicoGreen
<i>Range</i>				
DNA	1–50 $\mu\text{g/ml}$	0.01–15 $\mu\text{g/ml}$	0.1–10 $\mu\text{g/ml}$	25–1000 pg/ml
RNA	1–40 $\mu\text{g/ml}$	NA	0.2–10 $\mu\text{g/ml}$	Minimal sensitivity
<i>Ratio of signal (DNA/RNA)</i>	0.8	400	2.2	> 100

in both crude cell lysates and purified preparations can be quantified (Labarca and Paigen, 1980). Because the assay quantifies a broad range of DNA concentrations—from 10 ng/ml to 15 $\mu\text{g/ml}$ —it is useful for the measurement of both small and large amounts of DNA (e.g., in verifying DNA concentrations prior to performing electrophoretic separations and Southern blots). The Hoechst 33258 assay is also useful for measuring products of the polymerase chain reaction (PCR) synthesis.

Upon binding to DNA, the fluorescence characteristics of Hoechst 33258 change dramatically, showing a large increase in emission at ~ 458 nm. Hoechst 33258 is non-intercalating and apparently binds to the minor groove of the DNA, with a marked preference for AT sequences (Portugal and Waring, 1988). The fluorochrome 4',6-diamidino-2-phenylindole (DAPI; Daxhelet et al., 1989) is also appropriate for DNA quantitation, although it is not as commonly used as Hoechst 33258. DAPI is excited with a peak at 344 nm. Emission is detected at ~ 466 nm, similar to Hoechst 33258.

Ethidium bromide is best known for routine staining of electrophoretically separated DNA and RNA, but it can also be used to quantify both DNA and RNA in solution (Le Pecq, 1971). Unlike Hoechst 33258, ethidium bromide fluorescence is not significantly impaired by high GC content. The ethidium bromide assay (with excitation at 546 nm) is ~ 20 -fold less sensitive than the Hoechst 33258 assay.

In addition to the advantages mentioned in the protocol itself (see Alternate Protocol 3), the PicoGreen assay protocol also minimizes the fluorescence contribution of RNA and single-stranded DNA (ssDNA). Although the Hoechst 33258-based method is not significantly affected by the presence of RNA when the assay is carried out in the recommended

high-salt buffer, Hoechst 33258 does exhibit a large fluorescence enhancement with ssDNA under these conditions. Furthermore, when the Hoechst 33258-based assay is carried out in TE alone (10 mM Tris·Cl/1 mM EDTA, pH 7.5, with no NaCl added), RNA contributes a significant fluorescence signal. Using the PicoGreen dsDNA quantitation reagent as described in Alternate Protocol 3, dsDNA can be quantitated in the presence of equimolar concentrations of ssDNA and RNA with minimal effect on the quantitation results.

Critical Parameters

For instruments requiring cuvettes, special care should be taken when cleaning or handling sample cuvettes. Similarly, the measurement surfaces in all spectrophotometric procedures should be kept clean of debris. Traditional fluorometers use cuvettes with four optically clear faces because excitation and emitted light enter and leave the cuvette through directly adjacent sides. Thus, fluorometric cuvettes should be held by the upper edges only. In contrast, transmission spectrophotometers use cuvettes with two opposite optical windows, with the sides frosted for easy handling. It is important to check that the optical faces of cuvettes are free of fingerprints and scratches. In addition, for accurate absorbance readings, spectrophotometer cuvettes must be perfectly matched.

Proteins in general have A_{280} readings considerably lower than nucleic acids on an equivalent weight basis. Thus, even a small increase in the A_{280} relative to A_{260} (i.e., a lowering of the A_{260}/A_{280} ratio) can indicate significant protein contamination. In addition, some commonly used buffer components absorb strongly at 260 nm and can cause interference if present in high enough concentrations. EDTA, for example, should not be present at ≥ 10 mM.

Sensitivity of the Hoechst 33258 fluorescence assay decreases with nuclease degradation, increasing GC content, or denaturation of DNA (Labarca and Paigen, 1980; Stout and Becker, 1982). Increased temperature of the assay solution and ethidium bromide contamination also decrease the Hoechst 33258 signal. Sodium dodecyl sulfate (>0.01% final concentration) also interferes with accurate readings (Cesarone et al., 1979). The pH of the assay solution is critical for obtaining maximum sensitivity and should be ~7.4 (Labarca and Paigen, 1980; Stout and Becker, 1982). At a pH <6.0 or >8.0 the background becomes much higher and there is a concomitant loss of fluorescence enhancement.

High-quality double-stranded DNA is recommended, although single-stranded genomic DNA also works well with the Hoechst 33258 assay. However, with very small fragments of DNA, the Hoechst 33258 dye binds to double-stranded DNA only. Thus, the assay will not work with single-stranded oligomers. Linear and circular DNA yield approximately the same levels of fluorescence (Daxhelet et al., 1989). When preparing DNA standards, an attempt should be made to equalize the GC content of the standard DNA and that of the sample DNA. In most situations, salmon sperm or calf thymus DNA is suitable. An extensive list of estimated GC content for DNA from various organisms is available (Marmur and Doty, 1962). Eukaryotic cells vary somewhat in GC content but are generally in the range of 39% to 46%. Within this range, the fluorescence per microgram of DNA does not vary substantially. In contrast, the GC content of prokaryotes can vary from 26% to 77%, causing considerable variation in the fluorescence signal. In these situations, the sample DNA should be first quantitated via transmission spectroscopy

and compared to a readily available standard (e.g., calf thymus DNA). Future measurements would then use calf thymus as a standard, but with a correction factor for difference in fluorescence yield between the two DNA types. For further troubleshooting information, see Van Lancker and Gheysens (1986), in which the effects of interfering substances on the Hoechst 33258 assay (and several other assays) are compared.

In the ethidium bromide assay, single-stranded DNA produces approximately half as much signal as double-stranded calf thymus DNA. Ribosomal RNA also gives about half the fluorescent signal as double-stranded DNA, and RNase and DNase both severely decrease the signal. Closed circular DNA also binds less ethidium bromide than nicked or linear DNA. Further critical parameters of the ethidium bromide assay are described by Le Pecq (1971).

With PicoGreen, dsDNA can be quantitated in the presence of equimolar concentrations of single-stranded nucleic acids with minimal interference. Table A.3D.8 shows the concentrations of RNA or ssDNA that, for a given dsDNA concentration, result in less than a 10% change in the signal intensity using the PicoGreen assay protocol. Fluorescence due to PicoGreen reagent binding to RNA at high concentrations can be eliminated by treating the sample with DNase-free RNase. The use of RNase A/RNase T1 with S1 nuclease will eliminate all single-stranded nucleic acids and ensure that the entire sample fluorescence is due to dsDNA.

Anticipated Results

The detection limit of absorption spectroscopy will depend on the sensitivity of the spectrophotometer and any UV-absorbing

Table A.3D.8 Sensitivity of the PicoGreen dsDNA Assay for Quantitating dsDNA in the Presence of Single-Stranded Nucleic Acids

[dsDNA]	[RNA]	Amount of RNA relative to dsDNA	[ssDNA]	Amount of ssDNA relative to dsDNA
1 µg/ml	10 µg/ml	10×	300 ng/ml	0.3×
500 ng/ml	500 ng/ml	1×	50 ng/ml	0.1×
10 ng/ml	100 ng/ml	10×	30 ng/ml	3×
5 ng/ml	50 ng/ml	10×	15 ng/liter	3×
100 pg/ml	1 ng/ml	10×	1 ng/ml	10×
50 pg/ml	500 pg/ml	10×	500 pg/ml	10×

^aFor several concentrations of dsDNA, the concentration of RNA or ssDNA is shown that results in no more than a 10% increase in the sample's signal intensity.

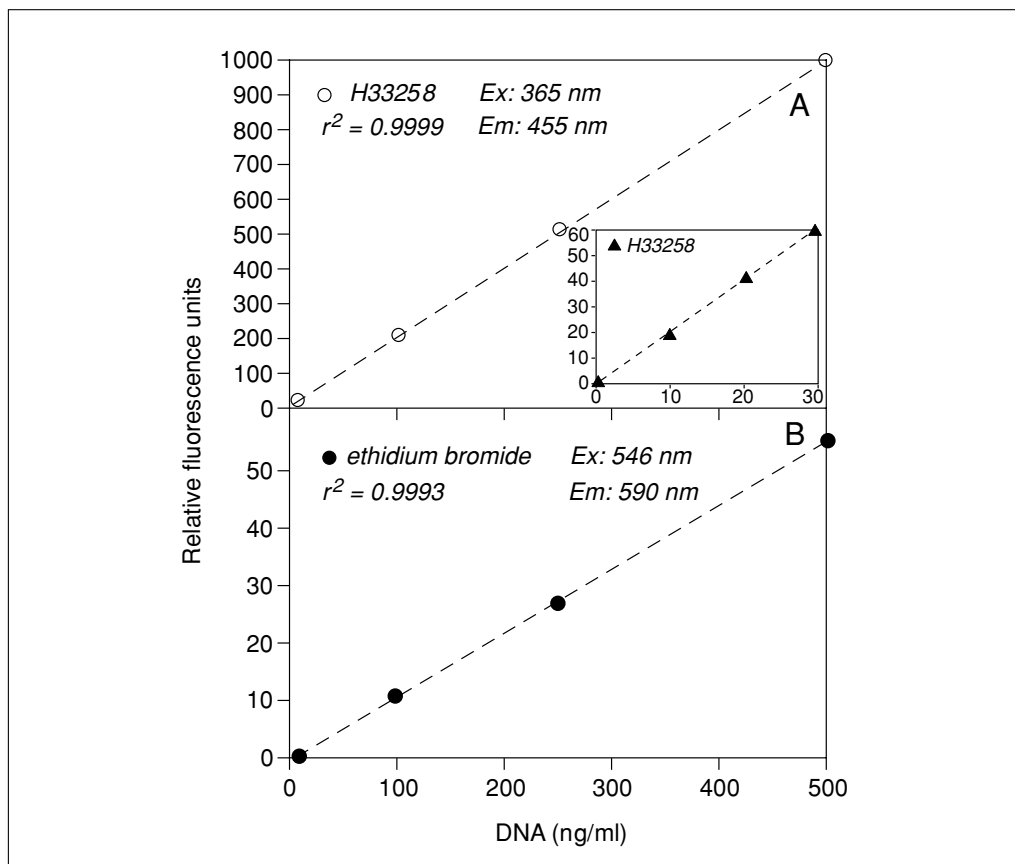


Figure A.3D.2 Fluorochrome H33258 (**A**) and ethidium bromide (**B**) DNA concentration standard curves. Assays were performed as described in the alternate protocols, at indicated excitation and emission wavelengths. The concentrations of H33258 and ethidium bromide were 0.1 and 5 $\mu\text{g/ml}$, respectively. Assays contained the indicated concentrations of calf thymus DNA standards suspended in a final volume of 2.0 ml. Inset shows low DNA concentration curve for the H33258 assay. Note that, under these conditions, H33258 produces ~ 20 times more relative fluorescence units than ethidium bromide. A Shimadzu RF-5000 scanning fluorescence spectrometer was used for both assays.

contaminants that might be present and the quantity of sample available to the investigator. The lower limit is generally ~ 0.5 to 1 μg nucleic acid for traditional spectrophotometers.

For the Hoechst 33258, ethidium bromide, and PicoGreen assays, a plot of relative fluorescence units or estimated concentration (y axis) versus actual concentration (x axis) typically produces a linear regression with a correlation coefficient (r^2) of 0.98 to 0.99 (Fig. A.3D.2). Table A.3D.7 provides a comparison of the sensitivities and specificities of the assays.

Time Considerations

The six assays described can be performed in a relatively short period of time. In a well-planned series of assays, 50 samples can be prepared and read in 1 hr.

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Key Reference

Labarca and Paigen, 1980. See above.

Contains a detailed description of the Hoechst 33258 fluorometric DNA assay.