
A QUANTIFLUOR™-ST Fluorometer Method for DNA Quantitation Using Hoechst 33258

INTRODUCTION

Quantitation of DNA is a prelude to many practices in molecular biology. Common techniques that use DNA, such as sequencing, cDNA synthesis and cloning, RNA transcription, transfection, nucleic acid labeling (e.g., random prime labeling, etc.) all benefit from a defined template concentration. Failure to produce results from these techniques can sometimes be attributed to an incorrect estimate of the DNA template used.

The concentration of a nucleic acid is most commonly measured by UV absorbance at 260nm (A_{260}). The average extinction coefficient for double-stranded DNA ($1 A_{260} = 50\mu\text{g/ml}$), single stranded DNA ($1 A_{260} = 33\mu\text{g/ml}$), or RNA ($1 A_{260} = 40\mu\text{g/ml}$) is used to quantitate the nucleic acid directly from the absorbance at this wavelength. For accurate results, absorbance should be in the range of 0.05– 0.10, which for a 1.0ml assay, requires 2.5–5.0 μg of dsDNA. For dilute nucleic acid samples, the solution to be measured should also be relatively free of other components that would add significantly to the absorbance at 260nm. Because of these limitations, alternate techniques have been sought that provide more sensitivity and are less variant to background absorbance.

One such alternative for reliable quantitation of DNA that significantly improves sensitivity and begins to address the issues of variance is fluorescence. As with the common practice of visualizing DNA in a gel with ethidium bromide, quantitation of DNA can be easily achieved in a fluorometer with the dye, Hoechst 33258, a bisbenzimidazole DNA intercalator that excites in the near UV (350nm) and emits in the blue region (450nm). Sensitivity of the Hoechst 33258 assay is approximately 10ng/ml when it is used in conjunction with the QUANTIFLUOR™-ST Fluorometer. This dye overcomes some of the

limits associated with quantitation of dsDNA by absorbance measurements.

MATERIALS REQUIRED

- QuantiFluor™-ST Fluorometer
- 10x10mm methacrylate cuvettes
- Minicell Adaptor Kit
- Hoechst 33258 stock dye solution
- 10X TNE buffer stock solution
- 0.45 μm filtered water

FACTORS TO CONSIDER

The AT content of a DNA sample affects Hoechst 33258-DNA fluorescence. Hence, it is important to use a standard similar to the samples you are testing. Calf Thymus DNA can often serve as a reference for most plant and animal DNA because it is double-stranded, highly polymerized, and is approximately 58% AT (42% GC). For bacterial DNA, a different standard may be needed because the AT content varies widely depending on the species.

- The conformation (supercoiled, relaxed, circular, linear) of plasmid DNA may result in different Hoechst 33258 binding efficiencies. Thus, it is important to select a standard with similar physical characteristics to your sample.
- Hoechst 33258 fluoresces only about half as much when it binds to single-stranded genomic DNA compared to when it binds to double-stranded genomic DNA. In addition, short pieces of single-stranded DNA will not normally cause Hoechst 33258 to fluoresce in proportion to their concentration.

- Buffers commonly used to extract DNA from whole cells have little or no effect on this assay.
- Low levels of detergent (<0.01% SDS) have little or no effect on this assay.
- Salt concentrations in the sample extract of up to 3 M NaCl do not affect this assay. For peak fluorescence, at least 200 mM NaCl is required for purified DNA, and 2.0 to 3.0 M is required for crude samples. In crude samples, higher salt concentrations appear to cause the dissociation of proteins from DNA, allowing the dye molecules to bind to DNA easier.
- RNA does not interfere significantly with the DNA assay because Hoechst 33258 does not normally bind to RNA. Under high salt concentrations, fluorescence from RNA is usually less than 1% of the signal produced from the same concentration of DNA

SOLUTION PREPARATION

Note: Hoechst 33258 is a possible carcinogen and possible mutagen. Wear gloves and a mask, and work under a fume hood.

Hoechst 33258 stock dye solution (1 mg/ml)

Dilute 1ml Hoechst 33258 (10mg/ml solution) with 9ml distilled, 0.45µm filtered water. Store in an amber bottle at 4°C for up to 6 months.

10X TNE buffer stock solution

12.11g Tris base [Tris (hydroxymethyl) aminomethane], MW = 121.14
3.72g EDTA, disodium salt, dihydrate, MW = 372.20
116.89g NaCl, MW = 58.44
Dissolve into 800ml of distilled water. Adjust pH to 7.4 with concentrated HCl. Add distilled water to 1000ml. Filter (0.45µm) before use. Store at 4°C for up to 3 months.

Note: The pH and NaCl concentration are essential for proper binding of the Hoechst reagent.

1X TNE

10ml 10X TNE
90ml distilled, 0.45 µm filtered water

2X Dye Solution (200ng/ml)

For 10–1000ng/ml final DNA concentration.
20µl Hoechst 33258 stock solution (1mg/ml)
100ml 1X TNE
Keep assay solution at room temperature. Prepare fresh daily. Do not filter once dye has been added

Calf thymus DNA standard

Prepare a 1mg/ml stock solution of calf thymus DNA in TE.
Gently tap the tube to mix thoroughly. Store at 4°C for up to 3 months.

PROTOCOL

Note: Accurate pipetting and thorough mixing are critical for reproducible results. However, take extreme care when mixing samples; do not introduce air bubbles. Air bubbles can cause scattering of light leading to inaccurate results. If air bubbles form, hold the upper portion of the cuvette in one hand and gently tap the bottom sides of the cuvette with your other hand to release bubbles.

1. Choose the assay range most suitable for your samples. For 10 to 1000ng/ml DNA standards (as shown in Table 1), prepare a series of DNA solution at 2X final concentration. Mix equal volume of the 2X DNA solution with the prepared 2X dye solution and place into disposable cuvettes or plastic test tubes for transfer to Minicell cuvettes. For blank controls, mix equal volume of 1X TNE with the 2X Dye Solution. Be sure to add enough volume into the cuvettes. The minimum volume is 2ml for 10x10mm cuvette and 50µl for Minicell cuvette.
2. Turn on the fluorometer and select the UV channel. Calibrate the instrument with the highest standard.
3. Prepare the unknown samples by diluting samples to desired volume. Add equal volume of the 2X dye solution used for standards and blank.

Table 1. DNA standard curve for 10x10 mm cuvette.

2X DNA solution concentration (ng/mL)	Volume of the 2X DNA solution (mL)	Volume of the 2X dye solution (mL)	Final DNA concentration in Hoechst Assay (ng/mL)
2000	1	1	1000
1000	1	1	500
500	1	1	250
200	1	1	100
100	1	1	50
50	1	1	25
20	1	1	10
0	1	1	blank

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CONTACT INFORMATION

Toll-Free: (800) 356-9526

Fax: (800) 356-1970

www.promega.com

Email: custserv@promega.com

Mailing Address:

Promega Corporation
2800 Woods Hollow Rd.
Madison, WI 53711 USA

GENERATING A STANDARD CURVE

Generating a standard curve verifies the linearity of the assay within a particular concentration range. It is recommended that you perform this at least once when working with a new instrument or performing the assay for the first time. Also, you may want to generate a standard curve every few weeks as a quality check on the standard, a reliability check on the instrument, and a consistency check on technique.

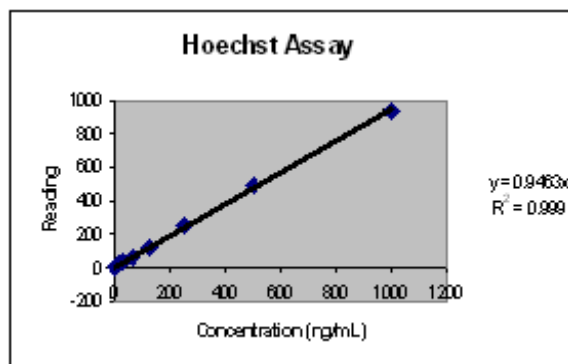


Figure 1. Calf Thymus DNA stained with Hoechst 33258 dye and fluorescence measured on the QuantiFluor™-ST Fluorometer.

Note: If the measured values near one end of the curve deviate consistently from the line, those values represent a nonlinear region. Sample concentrations should be adjusted to stay within the linear region of the assay.