

A QuantiFluor™-P Method for DNA Quantitation Using Hoechst 33258

INTRODUCTION

DNA quantitation is a necessary step for many life science research protocols. Common DNA techniques, such as sequencing, cDNA synthesis and cloning, RNA transcription and transfection, all benefit from a defined template concentration. An inaccurate estimation of the amount of DNA template may cause these techniques to fail.

DNA concentration is often measured by UV absorbance at 260 nm ($1A_{260} = 50 \mu\text{g/mL}$) in a 1 cm path length cuvette. This method, however, is not as accurate as DNA quantitation using fluorescent dyes. The *QuantiFluor™-P* can be used for DNA quantitation along with Hoechst 33258, a bisbenzimidazole DNA intercalator that excites in the near UV (350 nm) and emits in the blue region (450 nm). Hoechst 33258 binds to the AT rich regions of double stranded DNA and exhibits enhanced fluorescence under high ionic strength conditions. Sensitivity of the Hoechst 33258 assay in combination with the *QuantiFluor™-P* is approximately 10 ng/mL DNA. The linear dynamic range extends over 3 orders of magnitude from 10 ng/mL to 1 $\mu\text{g/mL}$ DNA.

MATERIALS REQUIRED

- *QuantiFluor™-P* Handheld Fluorometer with UV optical configuration
- 10 x 10 mm Methacrylate fluorescence cuvettes
- Calf Thymus DNA Standard
- Hoechst 33258, 10 mg/mL
- 10X TNE buffer stock solution
- 0.45 μM filtered water
- 1X TE buffer

Note: The minicell is not recommended for this application

FACTORS TO CONSIDER

1. 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 species.
2. The conformation (ie. 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. The most stable form is linear.
3. 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.
4. 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.
5. Salt concentrations 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 NaCl for crude samples. In crude samples, higher salt concentrations appear to cause the dissociation of proteins from DNA, allowing the dye molecules to bind easier to DNA.

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

10X TNE buffer stock solution

12.11 g Tris base [Tris (hydroxymethyl) aminomethane], MW = 121.14

3.72 g EDTA, disodium salt, dihydrate, MW = 372.20

116.89 g NaCl, MW = 58.44

Dissolve into 800 mL of distilled water. Adjust pH to 7.4 with concentrated HCl. Add distilled water to 1000 mL. Filter (0.45 μ m) 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

Dilute 10 mL 10X TNE with 90 mL distilled, 0.45 μ m filtered water.

To prepare a 2X Dye Solution (200 ng/mL; for 10-1000 ng/mL final DNA concentration)

Dilute 20 μ L Hoechst 33258 stock dye solution (1 mg/mL) with 100 mL 1X TNE. Keep assay solution at room temperature. Prepare fresh daily. Do not filter once Hoechst dye has been added. Protect the 2X Dye solution from light.

Calf Thymus DNA standard

Prepare a 1 mg/mL stock solution of Calf Thymus DNA in 1X TE.

Gently tap the tube to mix thoroughly. Store at 4°C for up to 3 months.

PROTOCOL FOR GENERATING A STANDARD CURVE

Generating a standard curve verifies the linearity of the assay within a particular concentration range.

- Prepare a 3-fold serial dilution of the Calf Thymus DNA in 1X TNE from 2 μ g/mL DNA to 20 ng/mL.
- Mix 1 mL of 2 μ g/mL DNA standard with 1 mL of 2X Dye Solution and add to a labeled 10 x 10 mm cuvette. The final concentration of DNA is now 1000 ng/mL.
- Repeat step 5.2 for each DNA standard.
- Prepare a blank sample by mixing 1 mL 1X TNE buffer with 1 mL 2x Dye Solution. Protect all standards from light before measuring.

Note: Accurate pipetting and thorough mixing is 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.

- Set-up the *QuantiFluor™-P* per instructions in the *Technical Manual*. Power up the instrument by pressing the [ON/OFF] button. Use the [A/B] button to toggle to the "UV" channel. Press [STD VAL] to program in the concentration of your calibration standard. We suggest calibrating with 1000 ng of DNA std. You may set the value of your calibration standard to 999. Use the up and down arrows to set the concentration value. Hold down the arrow key to activate faster scrolling. When ready, press the [CAL] button to start the calibration.

The *QuantiFluor™-P* will measure one blank sample and one standard sample. After the calibration, you may press the [DIAG] button to check the full scale of your standard and blank. For best results, the %FS-Std should be greater than 3-fold of the %FS-Blk. If you cannot achieve a %FS-Std that is at least 3-fold of the %FS-Blk, contact our technical support staff.

Measure the fluorescence of the remaining standards to generate a standard curve of fluorescence versus DNA concentration. The standard curve provides an easy way to check the linearity of your calibration, but it not necessary to run a standard curve every time you perform the assay. The *QuantiFluor™-P* will automatically subtract the blank sample from each measurement. Figure 1 illustrates the fluorescence values (Y-axis) and DNA (ng/mL) (X-axis).

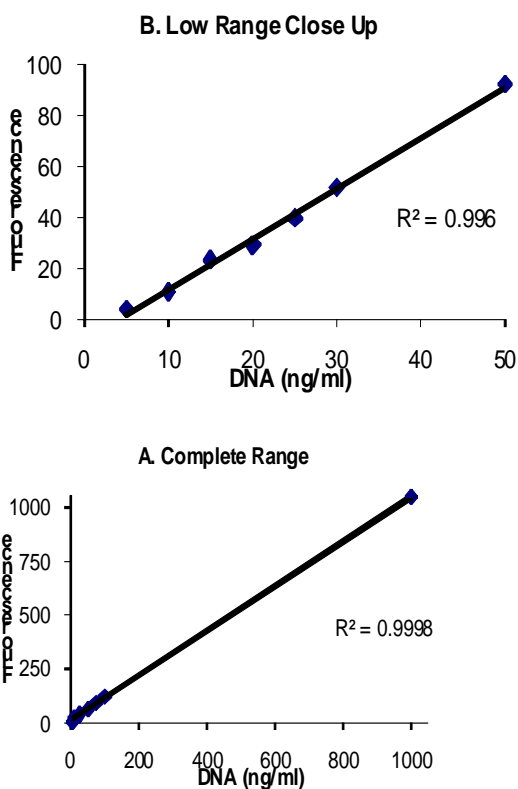


Figure 1. Complete range (A), and low range close up (B) Calf Thymus DNA stained with Hoechst 33258 dye and fluorescence measured on *QuantiFluor™-P* Handheld Fluorometer.

SAMPLE ANALYSIS

Dilute the experimental DNA solution in 1X TE to a final volume of 1 mL and add 1 mL of the 2x Dye Solution (prepared in section 4.1) to achieve a final volume of 2.0 mL. You may use two or three different dilution factors for a given sample.

Measure the fluorescence of each sample using the same calibration conditions as used to generate the standard curve (as in section 5).

The values reported by the *QuantiFluor™-P* represent the actual DNA concentration of the diluted sample. Remember to back-calculated each dilution to determine the original concentration.

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