A 20/20^n Method for DNA Quantitation Using

Hoechst 33258

1. INTRODUCTION

Quantitation of DNA is an important step for many practices in molecular biology. The concentration of a nucleic acid is most commonly measured by UV absorbance at 260 nm (A_{260}). Absorbance methods are limited in sensitivity due to the high level of background interference.

Hoechst 33258, a bisbenzimide DNA intercalator, provides a fluorometric alternative that is more sensitive than UV absorbance methods. Hoechst 33258 excites in the near UV (350 nm) and emits in the blue region (450 nm). Sensitivity of the Hoechst 33258 is better than 10 ng/mL when it is used in conjunction with the UV Fluorescent Module and the 20/20^n. (See Figure 1.)

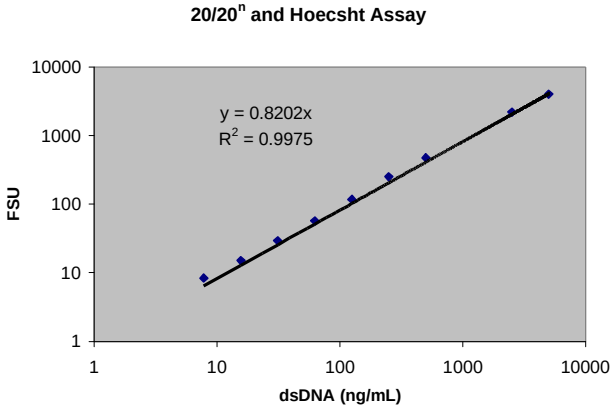

Figure 1. dsDNA and Hoechst dye analyzed on the 20/20^n. 10 µg/mL DNA was serially diluted in 1xTNE before the addition of 2x Hoechst Dye working solution. After a 5 minute equilibration period, 100 µL of each sample was transferred to a minicell cuvette and read using the UV Fluorescent Module.

2. MATERIALS REQUIRED

- 20/20^n Luminometer (P/N 2030-000)
- UV Fluorescent Module (P/N 2030-040)
- Minicell cuvettes (P/N 7000-950)
- Hoechst 33258 10 mg/mL (Molecular Probes H3569)
- 10X TNE buffer stock solution
- 0.45 µm filtered water

3. FACTORS TO CONSIDER

3.1 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.

3.2 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.

3.3 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.

3.4 Buffers commonly used to extract DNA from whole cells have little or no effect on this assay.

3.5 Low levels of detergent (<0.01% SDS) have little or no effect on this assay.

3.6 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.

3.7 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.

4. REAGENT PREPARATION

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

4.1 Hoechst 33258 stock dye solution (1 mg/mL):
Dilute 1 mL Hoechst 33258 (10 mg/mL solution) with 9 mL distilled, 0.45 µm filtered water.
Store in an amber bottle at 4^0C for up to 6 months.

4.2 10X TNE buffer stock solution:
Dissolve into 800 mL of distilled water:
12.11 g Tris base [Tris (hydroxymethyl) aminomethane], MW = 121.14
3.72 g EDTA, disodium salt, dihydrate, MW = 372.20
116.89 g Sodium chloride, MW = 58.44
Adjust pH to 7.4 with concentrated HCl.
Add distilled water to 1000 mL.
Filter (0.45 µm) before use.
Store at 4^0C for up to 3 months.

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

4.3 1X TNE: Dilute 10 mL 10X TNE with 90 mL distilled, 0.45 µm filtered water.

4.4 To prepare a 2X Dye Solution (200 ng/mL) for 10-1000 ng/mL final DNA concentration: Dilute 20 µL Hoechst 33258 stock solution (1 mg/mL) with 100 mL 1X TNE. Keep assay solution at room temperature. Prepare fresh daily. Do not filter once dye has been added.

4.5 Calf thymus DNA standard:
Prepare a 1 mg/mL stock solution of calf thymus DNA in TE. Gently tap the tube to mix thoroughly. Store at 4^0C for up to 3 months.

5. INSTRUMENT SET-UP

5.1 With the 20/20^n powered OFF, insert the UV Fluorescent Module according to the operating instructions.

5.2 Turn ON the 20/20^n. Allow the 20/20^n a 5-minute warm up period before calibration.

6. PROTOCOL

6.1 Prepare the standard solution. Dilute 1 mg/mL stock solution of DNA to a concentration of 2 µg/mL in 1xTNE. Add an equal volume of the 2 µg /mL DNA to 2x Hoechst dye working solution, prepared in step 4.4. Mix well in a microcentrifuge tube.
NOTE: 1000 ng/mL DNA is an acceptable standard for most DNA concentrations. To optimize the accuracy, use a standard that is at or near the concentration of a typical sample. For example, if a typical sample is 300 ng/mL DNA, use a standard of 500 ng/mL DNA. The standard should be at or above 100 ng/mL.

6.2 Prepare the blank solution. Add an equal volume of the sample buffer (usually 1xTNE without DNA) to 2x Hoechst dye working solution in a separate microcentrifuge tube.

6.3 Prepare the samples. Add equal volumes of the sample to 2x Hoechst dye working solution in a separate microcentrifuge tube.

NOTE: Do not mix samples, standard or blank solution with the Hoechst dye in the minicell cuvette.

6.4 Transfer 100 µL of each sample, standard, and blank solution to a minicell cuvette. Incubate for 2-5 minutes at room temperature, protected from light**.**
NOTE: Do not introduce air bubbles in the minicell cuvette. Air bubbles cause erroneous readings.

6.5 Touch “Calibrate” and select ng/mL for the unit of measure.

6.6 Using the number pad, enter 1000 for the standard concentration.

6.6.1 Insert the minicell cuvette containing the blank solution into the Fluorescent Module. Touch "OK" to start the calibration readings.

6.6.2 Insert the minicell cuvette containing the standard solution into the Fluorescent Module. Touch "OK" to finish the calibration reading.

6.7 Measure the sample solutions. Insert the minicell into the Fluorescent Module and touch "Measure Direct Fluorescence." The concentration of DNA in the minicell cuvette will appear after a 5-second integration.

NOTE: It is not necessary to run a standard curve after calibration. All subsequent readings will report in ng/mL final DNA concentration. Remember the final concentration is half of the sample concentration because of the addition of the dye.

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