

A TD-700 Laboratory Fluorometer Method for DNA Quantitation Using Hoechst 33258



1. 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 260 nm (A_{260}). The average extinction coefficient for double-stranded DNA ($1A_{260} = 50 \mu\text{g/mL}$), single stranded DNA ($1A_{260} = 33 \mu\text{g/mL}$), or RNA ($1A_{260} = 40 \mu\text{g/mL}$) 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 1.0 mL 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 260 nm. 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 (350 nm) and emits in the blue region (450 nm). Sensitivity of the Hoechst 33258 assay is approximately 1 ng/mL. This dye

overcomes some of the limits associated with quantitation of dsDNA by absorbance and can be used in the Turner Designs TD-700 Laboratory Fluorometer.

2. MATERIALS REQUIRED

- TD-700 Laboratory Fluorometer with standard PMT (P/N 7000-009)
- Near UV Lamp (P/N 10-049)
- Excitation filter (P/N 10-069R)
- Emission filter (P/N 10-110RC)
- 10mm x 10mm Methacrylate fluorescence cuvettes (P/N 7000-959)
- Hoechst 33258 stock dye solution
- 10X TNE buffer stock solution
- 0.45 μm filtered water

3. FACTORS TO CONSIDER

3.1 The AT% 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% varies widely depending on 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. The most stable form would be a linear one.

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 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.

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. SOLUTION 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°C 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°C for up to 3 months.

***NOTE:** The pH and NaCl concentration are essential for the Hoechst reagent to bind properly.

4.3 Low range assay solution (for 10-500 ng/ml final DNA concentration):

Dilute 10 µl Hoechst 33258 stock solution (1 mg/ml) with 10 ml 10X TNE and 90 ml Distilled, 0.45 µm filtered water.

Keep assay solution at room temperature. Prepare fresh daily. Do not filter once dye has been added.

4.4 High range assay solution (for 100-5000 ng/ml final DNA concentration):

Dilute 100 µl Hoechst 33258 stock solution (1 mg/ml) with 10 ml 10X TNE and 90 ml Distilled, 0.45 µm filtered water.

Keep assay solution at room temperature. Prepare fresh daily. Do not filter once dye has been added.

4.5 1X TNE: Dilute 10 ml 10X TNE with 90 ml Distilled, 0.45 µm filtered water.

4.6 Calf Thymus DNA Standard:

0.25 Unit (12.5 µg/ml) Calf thymus DNA
Dilute with 1X TNE to desired concentration.
Gently tap the tube to mix thoroughly.
Store at 4°C for up to 3 months.

5. 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.

5.1 Choose the assay range most suitable for your samples. If the low range assay (10 to 500 ng/mL final DNA concentration) is selected, prepare 2 ml of 100 ng/mL DNA by adding 2 µl 100 µg/ml DNA to 2 ml low range assay solution prepared in 4.3. If the high range assay (100 to 5000 ng/mL final DNA concentration) is selected, prepare 2 ml of 1000 ng/ml DNA by adding 20 µl 100 µg/ml DNA to 2 ml high range assay solution prepared in 4.4.

5.2 Check that lamp and filters are installed correctly according to the TD-700 Fluorometer Operating Manual.

5.3 Turn on fluorometer and allow to warm up for 10 minutes (600 seconds).

5.4 Set-up parameters and calibrate fluorometer (refer to your Operating Manual for detailed instructions). Calibration with more than 1 standard is recommended (see section 6.).

5.5 Measure the fluorescence of unknown samples by adding 2 µl unknown sample to 2 ml assay solution used for standards and blank.

6. 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.

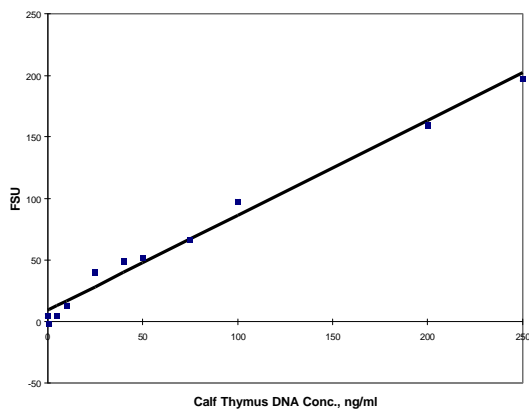


Figure 1. Calf Thymus DNA stained with Hoechst 33258 dye and fluorescence measured on Turner Designs TD-700 Laboratory Fluorometer.

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.

8. ABOUT TURNER DESIGNS, INC.

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