INTRODUCTION

DNA quantitation is an essential step for several life science research protocols. RNA transcription and transfection, sequencing, cDNA synthesis and cloning are all examples of common DNA techniques which 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 µg/mL) in a 1 cm path length cuvette. However, greater accuracy can be achieved for quantitating DNA using fluorescent dyes such as Hoechst 33258, a bisbenzimide 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. Fluorescence from Hoechst 33258 is proportional to the concentration of DNA in the sample and this fluorescence can be detected using the Trilogy with a Hoechst 33258 Module.

MATERIALS REQUIRED

- Trilogy Laboratory Fluorometer P/N 7200-002
- Hoechst 33258 Trilogy Module P/N 7200-030 or P/N 7200-030-W designed for quartz cuvettes
- 10 x 10 mm Methacrylate fluorescence cuvettes P/N 7000-959 or 12 x 75 mm Borosilicate Glass Disposable Culture Tubes P/N 10-029A
- Calf Thymus DNA Standard
- Hoechst 33258, 10 mg/mL Thermo Fisher Cat #H3569
- 10X TNE buffer stock solution
- 0.45 micron filtered water or 0.45 micron glass fiber filters
- 1X TE buffer

FACTORS TO CONSIDER

- Calf Thymus DNA is double-stranded, highly polymerized, and is approximately 58% AT (42% GC) so it can often serve as a reference for most plant and animal DNA. A different standard may be needed for bacterial DNA, since the AT content varies widely depending on species.

- It is important to select a standard with physical characteristics similar to your sample considering the conformation (i.e. Supercoiled, relaxed, circular, and linear) of plasmid DNA may result in different Hoechst 33258 binding efficiencies. The most stable form is linear.

- 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.

- A buffer is a solution that prevents any changes in pH when acid or alkali is presented. Buffers commonly used to extract DNA from whole cells have little or no effect on this assay. Low levels of detergent (<0.01%SDS) also have little or no effect on this assay.

- 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. A dissociation of proteins from DNA becomes present in crude samples when there are higher salt concentrations. This allows the dye molecules to bind easier to DNA.

- Hoechst 33258 does not normally bind to RNA, therefore RNA does not interfere significantly with the DNA assay. Fluorescence from RNA is usually less than 1% of the signal produced from the same concentration of DNA when it is under high salt concentrations.

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 (1000 µg/mL)
Dilute 1 mL Hoechst 33258 (10 mg/mL solution) with 9 mL distilled, 0.45 µm filtered water. This solution should be stored in a darkened container at 4º C and is good for up to 6 months with proper storage.

10X TNE buffer stock solution
Dissolve into 800 ml of deionized water:

- 12.11 g Tris base [Tris (hydroxymethyl) aminomethane] (MW = 121.4);
- 3.72 g EDTA, disodium salt, dehydrate, (MW = 372.20);
- 116.89 g Sodium chloride, (MW = 58.44)

Adjust the pH of the solution to 7.4 with concentrated HCl and bring the total volume of the solution to 1000 ml using deionized water.

This solution can be stored at 4º C and is good for up to 3 months with proper storage.

NOTE: Filter through a 0.45 µm glass fiber filter before use; accurate pH and NaCl concentrations are crucial for proper binding of the Hoechst reagent.

1X TNE solution
Dilute 10 ml 10X TNE with 90 ml deionized water. Filter through a 0.45 µm glass fiber filter before use,

2X Dye Solution (0.2 µg/ml)
Dilute 0.02 mL Hoechst 33258 stock solution (1000 µg/ml) with 100 ml of 1X TNE solution. Assay must be kept in room temperature. Prepare fresh daily. Do not filter once dye has been added. The 2X dye solution must be protected from light exposure.

Calf Thymus DNA Standard

Prepare a 1000 µg/ml stock solution of Calf Thymus DNA in 1X TNE. Gently tap the tube to mix thoroughly. This solution can be stored at 4º C and is good for up to 3 months with proper storage.

PROTOCOL FOR GENERATING A STANDARD CURVE

Linearity for this assay is verified by preparing a 3-fold serial dilution of the Calf Thymus DNA in 1X TNE from 2 µg/mL to 0.02 µg/mL DNA, thereby generating a standard curve within a particular concentration range. Use the following protocol to check linearity:

1) Dilute Hoechst stock dye solution (1000 µg/mL) in 1X TNE to the following concentrations: 2 µg/mL, 0.2 µg/mL, and 0.02 µg/mL
2) Mix 1 mL of 2 µg/mL DNA standard with 1 mL of 2X Dye Solution and add to a labeled 10 x 10 mm cuvette or 12 x 75 mm round bottom test tube. The final concentration of DNA is now 1 µg/mL.
3) Repeat step 2 for each DNA standard made in step 1
4) Prepare a blank sample by mixing 1 mL 1X TNE buffer with 1 mL 2X Dye Solution. Be sure that all standards are protected from light exposure before measuring
5) Insert the Hoechst module into the Trilogy and power up the Trilogy
6) Select the UV Module Selection as indicated by the GUI Selection on the Hoechst Module's label
7) Read the Blank and all three standards and record the RFU values in a notebook or Excel spreadsheet
8) Plot the standard concentrations versus blank-corrected RFU values to generate your calibration curve

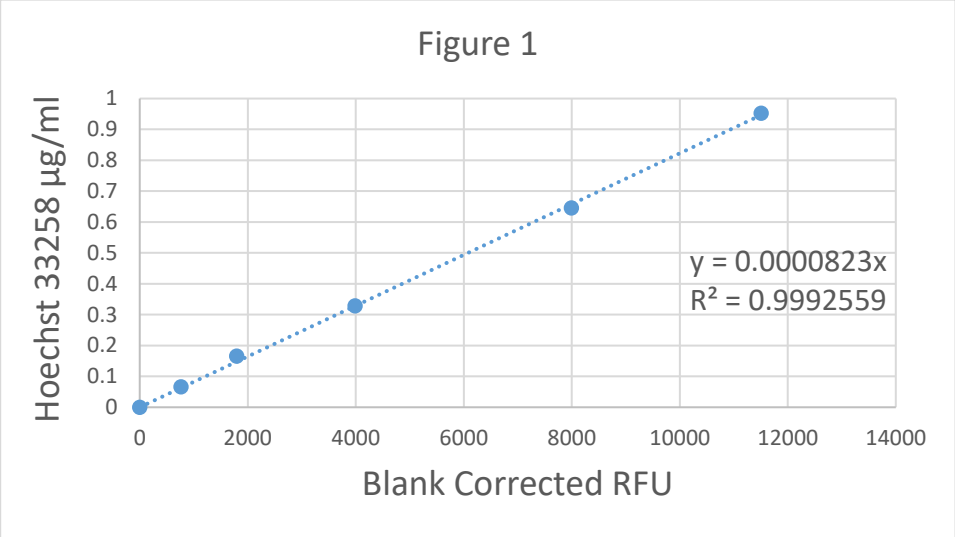
Alternatively, you may calibrate the Trilogy using your DNA calibration standards. To Calibrate the Trilogy:

1) Run through steps 1 – 6 from the protocol above
2) Press the Calibrate button
3) Follow calibration instructions to calibrate the Trilogy using your calibration standards
4) Name and save your calibration

NOTES:

- Accurate pipetting and thorough mixing is crucial for accurate end results.
- Be careful to not trap air bubbles in solution when mixing samples; they can result in light scatter and interfere with the fluorescence response decreasing accuracy of results. If air bubbles form, hold the upper portion of the cuvette with one hand while carefully and gently tapping the bottom of the cuvette with the other hand.
- It is not necessary to run a standard curve every time you perform the assay.

- The above protocol for generating a standard curve is recommended, however, users can generate a standard curve using as many standards as desired. Figure 1 below illustrates a standard curve using 5 standard solutions ranging from 0 to ~1 µg/mL.



SAMPLE ANALYSIS

1) Obtain a DNA sample solution.
2) Dilute it using 1X TNE to a final volume of 1 ml.
3) Add 1 ml of the 2X Dye Solution to achieve a final volume of 2.0 ml - you may use two or three different dilution factors for a given sample.
4) Measure the fluorescence of each sample using the Trilogy.
5) Either calculate the DNA concentration of the sample using your calibration curve or, if the calibration was stored on the Trilogy, the resulting DNA concentration will be displayed on the Trilogy's screen.