Fluorescent DNA Quantitation Kit

Instruction Manual

Catalog Number 170-2480

BIO-RAD

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Section 1
Introduction

It is often necessary to determine the concentration of DNA in solution. Numerous techniques, such as molecular cloning, fluorescence DNA sequencing, and DNA amplification require accurate measurement of DNA. The most common method for determining DNA concentration is by UV absorbance at 260 nm. However, this method has limitations. Contaminating products such as RNA and proteins absorb at the same wavelength as the DNA, thus generating inaccurate concentrations.

The fluorochrome Hoechst 33258 (bisbenzimide) is a sensitive and simple method for quantifying DNA.[1,2] The Hoechst 33258 dye binds to the minor groove of DNA with a preference for AT sequences.[2,3] Upon binding to DNA, the efficiency and the maximum wavelength of the fluorescence output of Hoechst 33258 shifts. The fluorescent changes can be measured using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preferential binding of Hoechst 33258 to DNA in the presence of high salt and neutral pH allows the DNA to be quantified in the presence of RNA, proteins (< 100 µg/ml)[4], nucleotides, and dilute buffer reagents.[1,2,5] The sensitivity of Hoechst 33258 allows double-stranded DNA to be quantified in the range of 20 ng to 10 µg (10 ng/ml to 5 µg/ml for a 2 ml assay), thus reducing the amount of DNA required for concentration measurements. The Hoechst 33258 dye also binds to single-stranded DNA with less efficiency than to double-stranded DNA and does not bind to small oligomers.[4] Linear and circular DNA will give similar fluorescence intensities.[3]

Calf thymus DNA is supplied in the kit to construct the standard curve from which unknown concentrations of DNA samples can be determined. Since Hoechst 33258 has preferential binding to AT sequences, it is advisable to use a standard that has similar AT content as that of the sample. In most cases, calf thymus DNA should be sufficient since it has a 58% AT content and is double-stranded.

Section 2
Kit Components

2.1 Contents

The Fluorescent DNA Quantitation Kit contains Hoechst 33258, calf thymus DNA, and 10x TEN assay buffer (100 mM Tris, 2 M NaCl, 10 mM EDTA, pH 7.4). There is sufficient reagent for 750 x 2.0 ml reactions or 7500 x 0.2 ml reactions.

Item	Concentration	Amount	Volume
Hoechst 33258 (bisbenzimide)	10 mg/ml	2.5 mg	0.25 ml
Calf Thymus DNA Standard	1 mg/ml	1 mg	1 ml
10x TEN Assay Buffer	10x	—	150 ml

2.2 Storage Conditions

The kit is shipped at room temperature. All components are guaranteed for 12 months from the date of purchase when stored at 4 °C and used as described in this manual.

Section 3
Preparation of Solutions

Note: Hoechst 33258 is a potential mutagen. Always wear gloves when handling this chemical.

1 mg/ml Hoechst 33258

10 mg/ml Hoechst 33258	100 µl
Sterile Water	900 µl

Mix above in a 1.5 ml microfuge tube. Store in the dark at 4 °C.

1 µg/ml Hoechst 33258

1 mg/ml Hoechst 33258	25.0 µl
10x TEN Assay Buffer	2.5 ml
Sterile Water	22.5 ml

Mix above in a 50 ml tube. This mix is enough for the 7 point standard curve and 5 samples, using 2 ml per sample. Adjust the volumes accordingly for more or less samples. Make fresh before use and place in the dark.

0.1 µg/ml Hoechst 33258

1 mg/ml Hoechst 33258	2.5 µl
10x TEN Assay Buffer	2.5 ml
Sterile Water	22.5 ml

Mix above in a 50 ml tube. This mix is enough for the 7 point standard curve and 5 samples, using 2 ml per sample. Adjust the volumes accordingly for more or less samples. Make fresh before use and place in the dark.

100 µg/ml DNA Standard

1 mg/ml Calf Thymus DNA	100 µl
10x TEN Assay Buffer	100 µl
Sterile water	800 µl

Mix above in a 1.5 ml microfuge tube. Store at 4 °C.

10 µg/ml DNA Standard

1 mg/ml Calf Thymus DNA	10 µl
10x TEN Assay Buffer	100 µl
Sterile water	890 µl

Mix above in a 1.5 ml microfuge tube. Store at 4 °C.

Section 4
Experimental Procedures

4.1 Fluorometer Set-Up

The Fluorescent DNA Quantitation Kit has been designed for use with any fluorometer system. The following section describes the procedure to be used with the Bio-Rad VersaFluor™ Fluorometer. If another instrument is used, refer to the instruction manual regarding specific operation details.

1. Turn on the fluorometer to allow instrument to warm up for at least 20 minutes. If the instrument is not fully warmed up, the reading may be irreproducible.
2. Insert the 360 nm excitation filter and the 460 nm emission filter into the fluorometer.
3. Set the gain to MED (medium) and adjust the range to read 00000.

4.2 Sample Measurement Guidelines

Follow the guidelines on the next page to insure accurate results.

- Warm up the instrument for at least 20 minutes
- Always use calibrated pipets to insure accurate pipetting
- Mix standards and sample completely by using a disposable transfer pipet
- Remove any air bubbles in the cuvette
- Hold fluorometer cuvettes by the upper edges since the cuvettes have four optically clear sides
- Clean the cuvette sides with a lint-free tissue
- Read all standards and samples at ambient temperature
- Keep the samples in the fluorometer only while making a reading to reduce photobleaching

4.3 Setting Up a Standard Curve

Calculating the concentration of unknown samples requires setting up a single point calibration or a standard curve. It is recommended that a standard curve be used to insure the linearity of the assay.

When setting up a standard curve, it is best to use DNA that has similar percent AT content to the samples being analyzed. The calf thymus DNA supplied in this kit is approximately 58% AT content and double-stranded. Determining the concentration of plasmid DNA may require generating a standard curve with that particular plasmid. This will maintain the same GC content and eliminate differences due to lower complexity of the plasmid sequence compared to the genomic calf thymus sequence. For analyzing samples from crude extracts, a higher salt concentration (2 M NaCl) may be needed in the assay buffer. The higher salt concentration is thought to dissociate proteins away from the DNA.[2]

There are two concentrations of Hoechst 33258 that are used depending on the range of DNA concentration. For determining the DNA concentration in the range of 100–5,000 ng/ml (200 ng–10 µg total DNA), use a Hoechst 33258 concentration of 1 µg/ml. For DNA in the range of

10–500 ng/ml (20–1,000 ng total DNA), use a Hoechst 33258 concentration of 0.1 µg/ml. Decide which standard curve is best suited for quantitating your samples or run both standard curves.

1. Set up a standard curve by first labeling seven 12.5 mm (OD) cuvettes (*e.g.*, 1–7). For a standard curve for DNA in the range of 200 ng–10 µg (100–5,000 ng/ml) refer to Table 1. For a standard curve for DNA in the range of 20 ng–1000 ng (10–500 ng/ml) refer to Table 2. For more accuracy, standards can be done in duplicate or triplicate.

   Note: If you are using a microplate fluorometer, refer to the Appendix for generating a standard curve.

2. Add 2.0 ml Hoechst 33258 dye to each cuvette (refer to Tables 1 and 2 for desired concentration range). Add the calf thymus DNA to cuvettes 1–6 as described in Table 1 or 2. Cuvette 7 is the blank and contains no DNA. Carefully mix the solutions using a disposable transfer pipet for each cuvette.

Table 1. 200 ng–10 µg DNA Range

Cuvette	Total DNA	DNA Stock Solution	DNA Volume	1 µg/ml Hoechst Dye
1	10,000 ng	1 mg/ml	10 µl	2 ml
2	5,000 ng	1 mg/ml	5 µl	2 ml
3	2,000 ng	1 mg/ml	2 µl	2 ml
4	1,000 ng	100 µg/ml	10 µl	2 ml
5	500 ng	100 µg/ml	5 µl	2 ml
6	200 ng	100 µg/ml	2 µl	2 ml
7	Blank	—	—	2 ml

Table 2. 20 ng–1000 ng DNA Range

Cuvette	Total DNA	DNA Stock Solution	DNA Volume	0.1 µg/ml Hoechst Dye
1	1000 ng	100 µg/ml	10 µl	2 ml
2	500 ng	100 µg/ml	5 µl	2 ml
3	200 ng	100 µg/ml	2 µl	2 ml
4	100 ng	10 µg/ml	10 µl	2 ml
5	50 ng	10 µg/ml	5 µl	2 ml
6	20 ng	10 µg/ml	2 µl	2 ml
7	Blank	—	—	2 ml

3. Zero the instrument with cuvette 7 (blank).
4. Set the range of the instrument by inserting the highest concentration cuvette (cuvette 1). Wait approximately 5-10 seconds for the detector to adjust to the light conditions. Set the range to 10,000 (Table 1 standard curve) or 1,000 (Table 2 standard curve). The fluorescence display should read the set value. Print or record the relative fluorescence unit (RFU) from the display.
5. The zero can be checked by placing cuvette 7 (blank) in the instrument. Re-zero the instrument if necessary.
6. Place cuvette 2 (5000 ng or 500 ng) into the instrument. Wait approximately 5–10 seconds for the detector to adjust to the light conditions. Record the RFU.
7. Repeat step 6 until all standard samples are read. Fluorescence measurements can be made continuously. It is not necessary to zero the instrument before each measurement.

4.4 Reading Unknown Samples

1. Add 2.0 ml Hoechst 33258 dye to each unknown sample cuvette. Use the same standard curve dye concentration (1 µg/ml or 0.1 µg/ml). For more accuracy, samples can be done in duplicate or triplicate.
2. Add the sample DNA to the cuvette. Carefully mix the solution using a disposable transfer pipet for each cuvette.

   Note: It is recommended you add ≤ 10 µl of DNA sample to the cuvette to reduce dilution of the dye.
3. Place a sample cuvette into the instrument. Wait approximately 5–10 seconds for the detector to adjust to the light conditions. Read or print the RFU number in the fluorescence display.
4. Repeat for all remaining samples. Fluorescence measurements can be made continuously. It is not necessary to zero the instrument before each measurement.

4.5 Data Analysis

The sample concentrations can be determined by comparing its relative fluorescence unit values with the standard(s).

1. Take an average of the samples if readings were in duplicate or triplicate.
2. From the data, prepare a calibration curve by plotting total DNA concentration (ng) versus relative fluorescence units (RFU).
3. To calculate the concentration of the unknown samples, determine the least squares regression equation for the line generated by the standard samples. The equation for a line is $y = mx + b$, where:

   y is the instrument reading (RFU)
   x is the DNA concentration (ng)
   m is the slope of the line
   b is the y-intercept

After determining the equation for the line, the unknown concentrations (x) can be solved. An example can be seen in Figure 1. In this example, 2 µl of an unknown sample had a relative fluorescence unit of 2000 and a calculated value of 4003 ng. The concentration can be calculated using the equation for the standard curve.

$y = 0.499x + 2.391$; where y is 2000
$2000 = 0.499x + 2.391$
$x = 4003$ ng

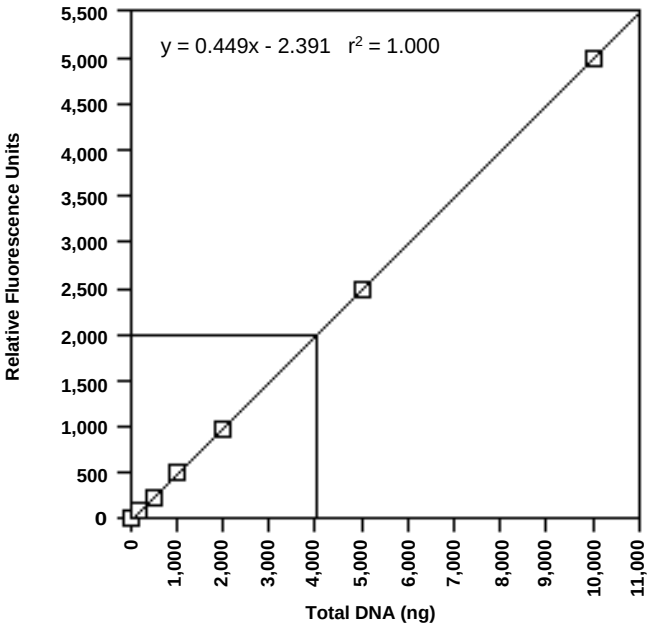

Fig. 1. Example plot of standard curve and linear equation.

4. Divide the total amount of DNA by the volume added to the cuvette to determine the concentration of the sample.

   Example: 4003 ng ÷ 2 µl = 2002 ng/µl

Section 5
Appendix

5.1 Microplate Fluorometer Guidelines

Use the following guide for making measurements on the Bio-Rad Fluoromark™ or other microplate fluorometers. Refer to the user manual for your fluorometer regarding specific operating instructions.

1. Measure the fluorescence for each solution using an excitation filter of 360–390 nm and an emission filter of 450–470 nm.

   Fluoromark Settings: Excitation filter: 355 nm, Emission Filter: 460 nm
2. To set the detection scale, insert the most concentrated solution made for the standard curve into the fluorometer. Adjust the gain to set the signal near the full scale detection.

   Fluoromark Settings: Gain: 18, Flash: 20
3. Refer to Table 3 for setting up a standard curve. Use 200 µl of 2 µg/ml Hoechst 33258 dye per well. For accuracy, standards and samples can be done in duplicates or triplicates.
4. Read the samples on the microplate fluorometer.

Table 3. 20–2000 ng DNA Range

Cuvette	Total DNA	DNA Stock Solution	DNA Volume	2 µg/ml Hoechst Dye
1	2000 ng	1 mg/ml	2 µl	200 µl
2	1000 ng	100 µg/ml	10 µl	200 µl
3	500 ng	100 µg/ml	5 µl	200 µl
4	100 ng	10 µg/ml	10 µl	200 µl
5	50 ng	10 µg/ml	5 µl	200 µl
6	20 ng	10 µg/ml	2 µl	200 µl
7	Blank	–—	–—	0

Section 6
References

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2. Labarca, C. and Paigen, K., *Anal. Biochem.*, 102, **344-352** (1980).
3. Daxhelet, G.A., Coene, M.M., Hoet, P.P., and Cocito, C.G., *Anal. Biochem.*, 179, **401–403** (1989).
4. Moe, D., Garbarsch, C., and Kirkeby, S., J. *Biochem. Biophys. Methods*, 28, **263–276** (1994).
5. Stout, D.L. and Becker, F.F., *Anal. Biochem.,*. 127, **302–307** (1982).

Section 7
Additional Supplies

Catalog Number	Product Description
	VersaFluor Fluorometer
170-2402	**VersaFluor Fluorometer 100/120/220**, includes a standard cuvette holder, 100 standard cuvettes, one excitation filter, and one emission filter
170-2420	**Optical Filter Excitation,** EX360/40 (340–380 nm)
170-2421	**Optical Filter Emission,** EM460/10 (455–465 nm)
	VersaFluor Disposable Cuvettes
170-2415	**Standard Cuvette,** 12.5 x 12.5 mm (OD) 4-sided optically clear disposable, polycarbonate, 3.5 ml, 100
	Fluoromark Microplate Fluorometer
170-6941	**Fluoromark Microplate Fluorometer 120V,** includes software, 5 excitation filters (355, 390, 485, 544 nm and time-resolved filter), and 5 emission filters (405, 460, 538, 590 nm, and time-resolved filter)
	Accessories
223-9522	**Disposable Transfer Pipet for the Standard Cuvette**
170-6963	**96-Well Fluorescence Microplate,** solid black, 25
170-6964	**96-Well Fluorescence Microplate,** solid black with clear bottoms, 25

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