Fluorescence Quantitation of Single-Stranded M13 DNA

Application Note #11
DyNA Quant® 200

Key Words: fluorescence DNA quantitation, M13 DNA, sequencing

Introduction

Determination of the concentration of recombinant M13 DNA is important for automated and manual DNA sequencing, gel electrophoresis analysis, molecular cloning, and site-directed mutagenesis. However, it is often difficult to obtain the quantities of highly purified M13 DNA needed for accurate measurement of concentration by UV absorption spectroscopy. Impurities such as proteins and RNA can prevent accurate spectrophotometric measurements of most routine M13 DNA preparations, particularly with single-stranded preparations. In addition, the concentration of DNA in such preparations can be below levels that can be accurately measured spectrophotometrically without using a significant portion of the DNA preparation.

Ethidium bromide fluorescence can be used to determine the DNA concentration of either double-stranded or single-stranded M13 DNA preparations [1]. However, ethidium bromide fluorescence can be sensitive to base-sequence composition as well as to DNA topology [2]. Thus fluorescence measurements must be made using a highly purified M13 DNA standard to calibrate the fluorometer or, if the fluorometer has been calibrated with a readily available DNA fluorometry standard, such as calf thymus DNA, the measurements must be normalized to compensate for the base-sequence composition and DNA topology differences between the M13 DNA and the standard DNA. Since ethidium bromide does not discriminate between RNA and DNA, any RNA contaminants in M13 DNA preparations can lead to over-estimations of DNA concentration.

All of these limitations can be overcome by using the DyNA Quant 200 Fluorometer to quantitate either single-stranded or double-stranded M13 DNA using the bis-benzimide (Hoechst 33258) fluorescence DNA assay [1,3,4,5]. The specificity of Hoechst 33258 (H 33258) for the minor groove of double-stranded DNA permits fluorescence determination of double-stranded DNA in the presence of RNA, nucleotides, and proteins [6,7,8]. Unlike ethidium bromide, H 33258 binding is not significantly affected by DNA topology [2,9]. Although H 33258 dye binding has been shown to be sensitive to base-sequence composition, the fluorescence behavior of H 33258 relative to DNAs of different base-sequence composition can easily be determined empirically or can be calculated [4,5]. More importantly, because of the specificity of H 33258 for the double-stranded B–DNA structure, it can bind to double-stranded hairpin structures that can form within single-stranded DNA under native conditions [10].

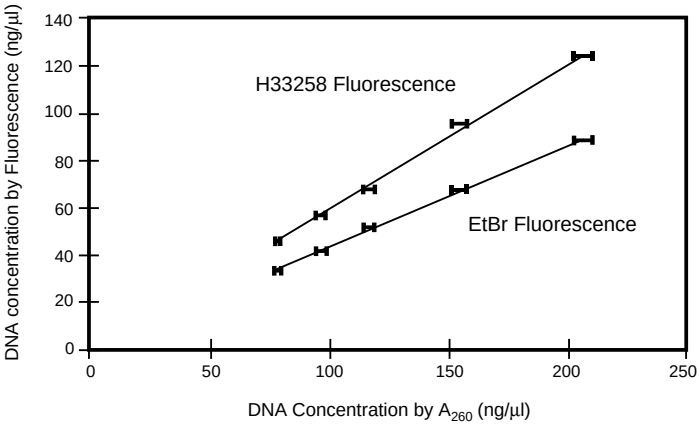

Figure 1. M13 (+) Strand DNA Quantitation: A_{260} versus H 33258 and ethidium bromide fluorescence. Purified M13 (+) strand DNA samples were measured by the H 33258/DNA fluorescence assay using the DyNA Quant 200 calibrated with the calf thymus DNA standard (upper curve). These same samples were also measured by ethidium bromide fluorescence using a filter fluorometer calibrated with the calf thymus DNA standard (lower curve). The concentration of DNA in the sample measured by A_{260} was plotted against the concentration of DNA measured by either H 33258 or ethidium bromide fluorescence (relative to calf thymus DNA). Linear regression was used to fit the data: $y = 0.6047x - 0.8145$; $r^2 = 0.9951$ for H 33258 and $y = 0.4274x + 1.0427$; $r^2 = 0.9967$ for ethidium bromide.

This report describes a simple method for the determination of a normalization factor to compensate for the H 33258 fluorescence differences between M13 (+) strand DNA and calf thymus DNA. The normalization factor accounts for both the single-stranded conformation and for the base-sequence composition differences between M13 (+) strand DNA and calf thymus DNA. Thus, accurate and precise quantitation of M13 (+) strand DNA can be performed with the DyNA Quant 200 fluorometer when using the calf thymus DNA standard.

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Results

Figure 1 shows the reproducibility and linearity of the H 33258 fluorescence in the presence of M13 (+) strand single-stranded DNA. As can be seen in the figure, the relationship between M13 (+) strand DNA concentration measured by UV absorption and by H 33258 fluorescence is linear (r^2 = 0.9951) with a slope of 0.605. Thus, M13 (+) strand binds approximately 60% as much H 33258 dye, per unit weight of DNA, as double-stranded calf thymus DNA. Since this relationship is linear, the slope of the line can be used as a normalization factor to correct measurements made with the DyNA Quant 200 calibrated with calf thymus DNA. For example, if the DNA concentration of a M13 (+) strand DNA preparation has been determined to be 100 ng/µl, using the DyNA Quant 200 calibrated with the calf thymus DNA standard, the actual concentration of M13 (+) strand DNA in the sample is 100 ng/µl ÷ 0.605 or 165 ng/µl.

For comparison, the experiment shown in Figure 1 was repeated using ethidium bromide for fluorescence determination of the same M13mp18 (+) strand DNA samples using a filter fluorometer calibrated with the calf thymus DNA standard at 100 ng/µl. The relationship between M13 (+) strand DNA concentration measured by UV absorption and M13 (+) strand DNA concentration measured by ethidium bromide fluorescence (Figure 1) is also linear (r^2 = 0.9967), with a slope of 0.427. This data indicates that ethidium bromide binds less efficiently to M13 (+) strand, relative to double-stranded calf thymus DNA, than does the H 33258 dye. As shown above for H 33258, the slope of the line for ethidium bromide binding in Figure 1 can be used as a normalization factor to correct ethidium bromide fluorescence measurements performed with a fluorometer calibrated with the calf thymus DNA standard. However, accurate measurements using ethidium bromide fluorescence can only be done with M13 DNA preparations that are free of RNA contaminants [1].

As an example of the use of the H 33258/DNA fluorescence assay for determining the concentration of M13 (+) strand DNA, a preparation of M13KO7 helper phage (27–1524–01), purified from *E. coli* strain NM522 (27–1525-01) using standard techniques [11], was quantitated by A_{260} and by the H 33258/DNA fluorescence assay. By A_{260}, the concentration of DNA was determined to be 175 ng/µl where as the concentration by the H 33258/DNA fluorescence assay was determined to be 28 ng/µl. This corresponds to 46 ng/µl after correcting using the normalization factor (0.605) determined above. The explanation for the discrepancy between the A_{260} and H 33258 fluorescence readings is apparent in the agarose gel shown in Figure 2; the single-stranded DNA preparation contains a significant amount of ribosomal RNA.

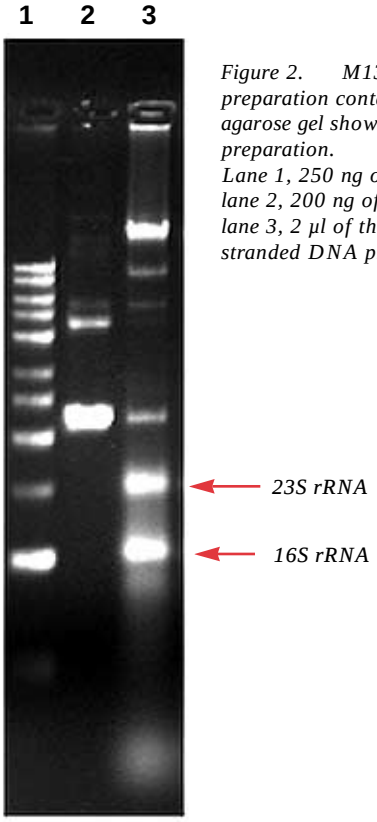

Figure 2. M13KO7 single-stranded DNA preparation contains ribosomal RNA. A 1 % agarose gel showing the single-stranded DNA preparation. Lane 1, 250 ng of the 1 kb ladder; lane 2, 200 ng of purified pGEM plasmid DNA; lane 3, 2 µl of the M13KO7 helper phage single-stranded DNA preparation.

Conclusion

In this note, we have determined that H 33258 fluorescence is linearly proportional to the concentration of M13 (+) strand DNA in a sample. We have determined the normalization factor (NF) for H 33258 fluorescence of M13 (+) strand DNA relative to our calf thymus DNA fluorometry standard (NF =1.65). Thus the DyNA Quant 200 fluorometer can be calibrated with a calf thymus DNA standard and be used to determine the M13 (+) strand DNA concentration accurately and precisely. Because RNA and protein can interfere with UV absorption and ethidium bromide fluorescence measurements, the H 33258/DNA fluorescence assay is the method of choice for determining M13 (+) strand DNA concentration in most routine preparations.

Materials and Methods

Equipment and Accessories

H 33258/DNA fluorescence quantitation was performed with the DyNA Quant 200 Fluorometer using the standard glass fluorometry cuvette [1,3,4]. Ethidium bromide fluorescence measurements were performed with a filter fluorometer using 365 ± 10-nm excitation and 605 ±10-nm emission bandpass interference filters and the standard glass fluorometry cuvette. The UV absorption measurements were made using a UV-visible spectrophotometer using quartz microcuvettes (100 µl volume). Electrophoresis was performed using an HE 99X horizontal gel electrophoresis unit.

Samples

M13mp18 (+) strand DNA (27-1546-01) at 200 ng/µl was diluted with TE, pH 8.0 to make 4 samples at 151, 113, 94, and 76 ng/µl.

UV Spectroscopic Measurements

The M13 DNA samples to be tested were diluted 1/50 in TE and scanned from 325 nm to 225 nm using a UV-visible spectrophotometer. The purity of the DNA samples was determined from the $A_{260}/_{280}$ ratios (>1.85). The concentrations were determined from the A_{260} reading using 40 µg/ml/A_{260} as the extinction coefficient. The concentrations of the DNAs were 78 ± 1, 96 ± 2, 116 ± 4, and 154 ± 1 for the diluted samples and 206 ± 2 ng/µl for the M13mp18 (+) strand DNA stock solution.

H 33258 Fluorescence Measurements Using the DyNA Quant

A stock of assay solution A (low DNA assay buffer) was made fresh. Calf thymus DNA in TE at 100 ng/µl (as determined by UV spectrophotometry) was used to calibrate the instrument.

Two ml of assay solution was added to the 4 ml cuvette which was then inserted into the instrument. After zeroing, the cuvette was removed and 2 µl of the calf thymus DNA standard was added. The solution was mixed using a disposable transfer pipette and the cuvette was inserted into the instrument for calibration at 100 ng/ml.

The DNA samples were measured, in duplicate, using the standard 2 ml assay [4,12] and the numbers were averaged.

Ethidium Bromide Fluorescence Measurements

The ethidium bromide assay solution was 0.5X TBE (44.5 mM Tris-Borate, pH 8.3, 1 mM EDTA) with 0.5 µg/ml ethidium bromide (17-1328-01). Calf thymus DNA in TE at 100 ng/µl (as determined by UV spectrophotometry) was used to calibrate the filter fluorometer.

Two ml of ethidium bromide assay solution was added to the 4 ml cuvette and inserted into the filter fluorometer. The fluorescence of the solution, in the absence of DNA, was set to zero. The cuvette was removed and 2 µl of the calf thymus DNA standard was added. The solution was mixed using a disposable transfer pipette and the cuvette was inserted into the filter fluorometer for calibration at 100 ng/ml.

The DNA samples were measured, in duplicate, as above and the numbers were averaged.

Electrophoresis

1. Running Conditions

Two µl of the indicated DNA sample was mixed with 4 µl of a loading solution (7.5% Ficoll, 0.0125% bromophenol blue in TE). The samples were loaded onto a 1% agarose gel in 1X TBE. Electrophoresis was for 3 hours at a constant 100 volts (about 50 mA).

2. Detection

The gel was stained for 1 hour at room temperature in 0.5 µg/ml ethidium bromide in 1X TBE. Gels were not destained prior to analysis.

References

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Ordering Information

Code No.	Item
80-6226-49	DyNA Quant 200 Fluorometer, 115V
80-6226-68	DyNA Quant 200 Fluorometer, 230V
80-6227-44	Glass Fluorometry Cuvette (DQ105)
80-6226-87	Hoechst 33258 Dye (100 mg, DQ201)
80-6227-06	Calf Thymus DNA Standard (250 µg, DQ202)
27-1546-01	M13mp18 (+) Strand DNA (25 µg)
17-1328-01	Ethidium Bromide Solution (10 mg/ml)
27-1524-01	M13KO7 helper phage
27-1525-01	*E. coli* strain NM522
80-6061-57	HE 99X Horizontal Gel Electrophoresis Unit