BioTek
Get a Better Reaction.

Application Note

Quantitation of ssDNA using OliGreen™ Fluorescent Stain

Several different techniques require the use of short synthetic oligonucleotide molecules, often referred to as primers. In each case, the use of appropriate concentrations of these molecules is paramount to the success of the experiment. Here we describe the use of OliGreen™ stain to quantitate ssDNA concentration using the BioTek FL600 fluorescence microplate reader.

Introduction

Synthetic oligonucleotides are used in a number of different molecular biology techniques, including DNA amplification (PCR), DNA sequencing, site directed mutagenesis, and in situ hybridization. These techniques require that precise concentrations of ssDNA oligonucleotide are present in the reactions. Unfortunately, the traditional absorbance based method of quantitation is not very sensitive, often requiring the use of concentrated samples.

The most commonly used technique to quantitate oligonucleotides and ssDNA is the measurement of their absorbance at 260 nm (A260). While this technique is easily performed, it is relatively insensitive, necessitating the use of concentrated stock solutions. Because of the large contribution of the nucleotide to the signal, premature synthesis terminations and/or nucleotides, common contaminants found in synthetic nucleic acid preparations, can result in erroneously high values. In addition, the actual amount required for specific reactions is well below the sensitivity limits of the absorbance procedure, requiring that large dilutions or the measurements of very small volumes from a concentrated stock be used. This often leads to errors that can result in poor experimental results. The ability to measure lower concentrations would thus eliminate the necessity to maintain concentrated stock solutions. Recent advances with intercalating fluorescent dyes such as ethidium bromide (1); Hoechst dye 33258 (2); and PicoGreen (3), have improved sensitivity to the picogram level, but are relatively specific for dsDNA, limiting their utility in the determination of ssDNA or oligonucleotide concentrations. Here we describe a fluorescent assay using OliGreen stain to quantitate ssDNA in the 96-well microplate based format using the BioTek FL600 fluorescence microplate reader in conjunction with KC4 data reduction software.

Materials and Methods

OliGreen was purchased from Molecular Probes (Eugene, OR). Black opaque stripwell 1x8 strips and frames, catalogue number 3914, were procured from Costar (Cambridge, MA). Synthetic oligonucleotides were obtained from several different sources; pUC forward primer was obtained from Promega Corp. (Madison, WI). Poly(dT) and poly(dA) oligonucleotides were from Pharmacia Biotech (Uppsala, Sweden). The ssDNA species M13mp18+ and purified sonicated dsDNA from salmon sperm were also obtained from Phamacia Biotech.
The linearity and detection limits of the OliGreen ssDNA assay were determined by making a series of dilutions ranging from 0.0 to 900 ng/ml of a ssDNA oligonucleotide (pUC forward primer) using TE, (10 mM Tris, 1 mM EDTA pH 7.5) as the diluent. For each concentration

 TEL: 888-451-5171 FAX: 802-655-7941 Outside the USA: 802-655-4740 E-mail: customercare@biotek.com

tested, replicates of four were determined. Linearity of the assay with large ssDNA polymers was assessed by dilution of M13 DNA from 0 to 1000 ng/ml as described above. The base selectivity of the stain was tested in a similar fashion except that dilutions of the homopolymers poly(dT) and poly(dA) were used instead of pUC forward primer. At each concentration replicates of four were determined. In order to assess the influence of dsDNA to the signal two equal portions of sonicated salmon sperm DNA stock solutions, where one was heat denatured at 95°C for 15 minutes and the other was maintained at room temperature, were diluted in parallel and stained with OliGreen fluorescent dye.

For all reactions, 100 ml aliquots of samples and standards were pipetted into microplate wells. After aliquoting the samples and standards, 100 ml of OliGreen working solution was added to each well. OliGreen working solution was prepared fresh by diluting OliGreenä concentrated reagent 1:200 with TE as described by the manufacturer. Samples were incubated for approximately 5 minutes at room temperature and their fluorescence determined using a Bio-Tek Instruments FL600 fluorescent plate reader with a 485 nm, 20 nm bandwidth, excitation filter and a 530 nm, 25 nm bandwidth emission filter. The sensitivity setting was varied as needed and the data collected from the top with a 3 mm probe using static sampling with a 0.35 second delay, 50 reads per well.

Results

As demonstrated in Figure 1, when stained with OliGreen, the ssDNA oligonucleotide (pUC forward primer) concentration shows a direct correlation with fluorescence. The fluorescence intensity was determined for pUC forward primer concentrations ranging from 0.0 to 900 ng/ml. Over this range the fluorescence intensity increased in a linear fashion. Using KC4 data reduction software (Bio-Tek Instruments), a linear regression equation describing the standard curve was generated. The high correlation coefficient (r2=0.9975) indicates that determinations can be made with a high level of confidence over the entire concentration range tested. When lower concentrations are observed (0-60 ng/ml) similar results are observed (Figure 1B). In terms of detection limits, concentrations as low as 1.25 ng/ml can be discriminated from a buffer only control. This represents quantities as small as 125 pg per well can be quantitated.

Several different physical properties of OliGreen fluorescent stain were examined. Base selectivity was investigated by measuring the fluorescence of two different ssDNA homopolymers in the presence of OliGreen. As seen in Figure 2, when poly(dT) and poly(dA) homopolymers are diluted in equal concentrations and stained with OliGreen, very different fluorescent signals are returned. OliGreen shows a significant increase in fluorescence in the presence of poly(dT), while very little signal with poly(dA) ssDNA oligonucleotides. Figure 3 demonstrates the enhancement of fluorescence by dsDNA. In parallel experiments equal concentration of either native or denatured sonicated salmon sperm DNA where assayed using OliGreen. The dsDNA (nondenatured) samples demonstrate almost three times the fluorescence as the denatured samples (Figure 3). Both experiments demonstrate a linear relationship between DNA concentration and fluorescence signal (Figure 3). The ability to quantitate large ssDNA polymers was tested using M13mp18+ DNA. This circular ssDNA molecule, 7,250 bases in length, is commonly used as a vector for sequencing unknown DNA. As demonstrated in Figure 4, increasing amounts of M13 ssDNA result in an increase in fluorescent signal. However, the relationship between fluorescence and ssDNA concentration is best described by a third order polynomial equation. Nevertheless, concentrations from 0 to 1000 ng/ml can be quantitated with a high degree of confidence, as indicated by the correlation coefficient (r2=0.9978).

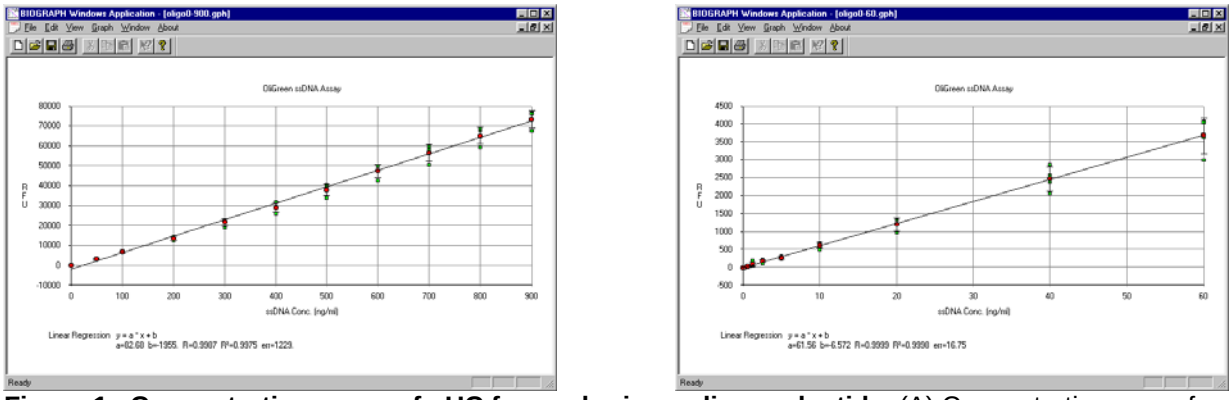

Figure 1. Concentration curve of pUC forward primer oligonucleotide. (A) Concentration curve from 0.0 to 900 ng/ml of pUC forward primer oligonucleotide with linear regression analysis. (B) Concentration curve from 0.0 to 60ng/ml of pUC forward primer oligonucleotide with linear regression analysis. Data indicated were recorded with a sensitivity setting of 125 and has been corrected by subtraction of a 0 ng/ml blank.

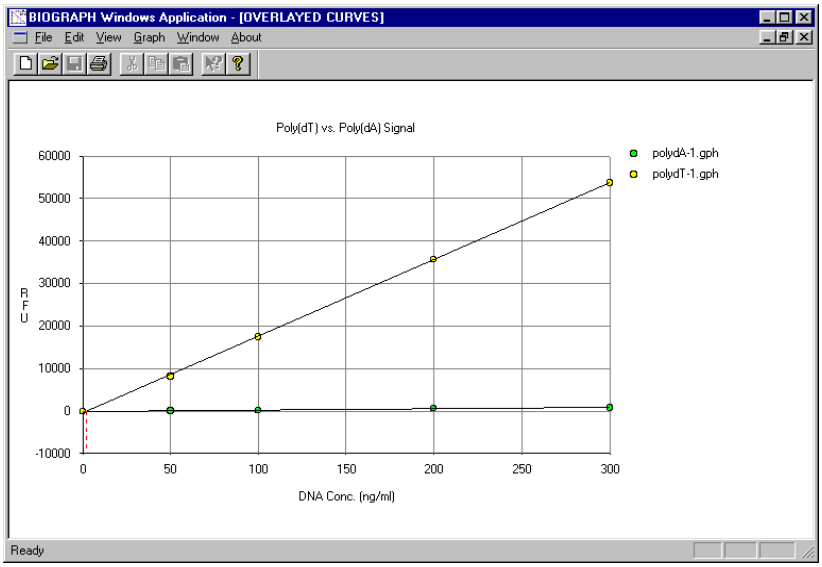

Figure 2. Comparison of OliGreen fluorescence with Poly(dA) and Poly(dT) homopolymers. Concentration curve from 0 to 300 ng/ml of either poly(dA) and poly(dT) homopolymers with linear regression analysis. Data indicated were recorded with a sensitivity setting of 110 and has been corrected by subtraction of a 0 ng/ml blank.

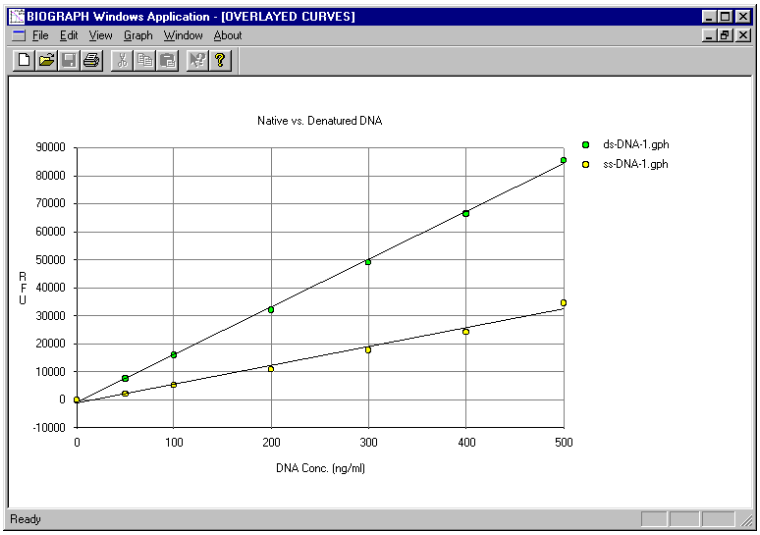

Figure 3. Comparison of OliGreen™ fluorescence with native and denatured DNA. Concentration curve from 0 to 500 ng/ml of either heat denatured or native purified salmon sperm DNA with linear regression analysis. Data indicated were recorded with a sensitivity setting of 120 and has been corrected by subtraction of a 0 ng/ml blank.

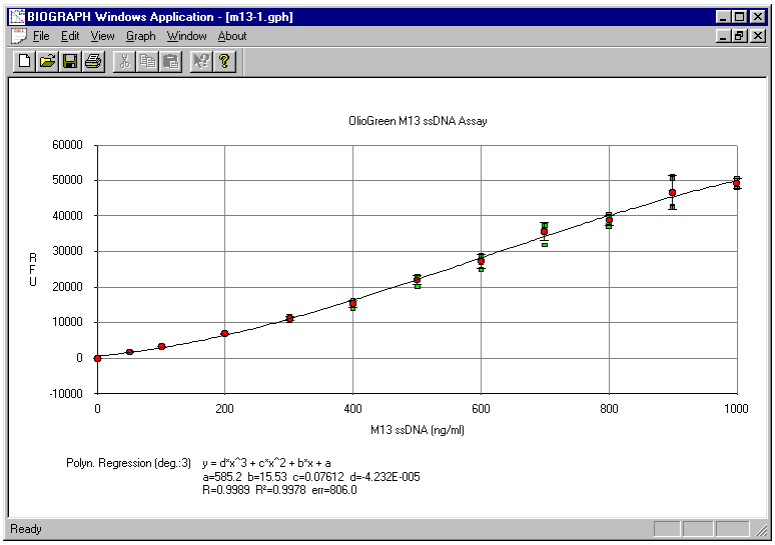

Figure 4. Concentration Curve of M13mp18+ viral DNA. Concentration curve of M13mp18+ ssDNA from 0 to 1000 ng/ml with polynomial curve fit of the data. Data indicated were recorded with a sensitivity of 120 and has been corrected by subtraction of a 0 ng/ml blank.

Discussion

Here we have demonstrated that the FL600 fluorescence microplate reader in conjunction with KC4 data reduction software can perform a fluorescent assay for ssDNA using OliGreen stain. This assay is linear over four orders of magnitude, from 0 to 1 mg/ml, without altering dye concentration. We have shown that OliGreen stain has a definite base selectivity. While most oligonucleotides have on average a similar percentage of each base, the possibility exists that specific oligonucleotides can be skewed in regards to their base composition. Homopolymers such as poly(dT), which is often used as a primer for first strand synthesis from poly(A)+ RNA, can result in different than expected concentrations if an incorrect oligonucleotide is used for a standard curve. For this reason, it is important that the standard curve be made with ssDNA that has a similar base composition as the unknown.

The preference of OliGreen for dsDNA can also result in a concentration discrepancy. OliGreen stain is most suitable for relatively short fragments, which can be produced by sonication of

genomic dsDNA. This process results in short fragments (600 bp or less) of dsDNA, which when denatured can be used for a number of purposes. Nevertheless, care must be taken to ensure that dsDNA has been denatured prior to measurement using OliGreen™. Likewise, M13 viral DNA, which is single stranded, but quite large, has areas that are complimentary that will form double stranded regions within itself. This results in a phenomenon where ssDNA and dsDNA exist simultaneously. This can be prevented by heat denaturing the sample immediately prior to the addition of OliGreen stain. The fluorescence enhancement with dsDNA must also be kept in mind when oligonucleotides with high propensity to form dimers are being quantified. These molecules may present a problem, particularly at high concentrations.

The OliGreen fluorescent assay for ssDNA offers a tremendous increase in sensitivity over existing assays. The use of OliGreen stain provides a 1000 fold increase in sensitivity over traditional absorbance methods. (1). In addition this assay does not require unique excitation and emission filters. In these experiments the standard 485/530 fluorescein filters were used with success. Because these filters are standard on the FL600, it is unnecessary to purchase any special filters. The ability of this assay to detect such small amounts of ssDNA (approximately 100 pg per well) makes it particularly useful. Diluted oligonucleotides can be measured directly rather than from a highly concentrated stock solution. This alleviates the loss of large amounts of precious sample when quantitating the sample, as well as reducing the likelihood of error caused by pipetting small amounts of concentrated sample. In addition, the linearity of the assay is also reported to be maintained in the presence of many contaminants commonly found in ssDNA preparations, including free nucleotides, urea, proteins, detergents, and agarose. It is important to note, however, that while linearity is maintained, signal intensity may be affected.

References

1. Maniatis T., E.F. Fritsch, and J. Sambrook (1982) Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
2. Labarca, C. and K. Paigen (1980) Anal. Biochem. 102:344-352.
3. PicoGreen dsDNA Quantitation Reagent and Kit Instruction, (1996) Molecular Probes, Eugene, OR.

Rev. 2-21-01