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Application Note

Quantitation of RNA with RiboGreen™ Stain using the FL600™

The quantitation of RNA samples is a prerequisite for many different techniques in molecular and cellular biology. Here we describe the use of RiboGreen™ fluorescent stain to quantitate RNA with the BioTek FL600 Fluorescence Microplate Reader.

Introduction

Quantitation of RNA samples is necessary for a number of different molecular biology techniques. These include measuring yields of synthetic ribonucleotides or in vitro transcribed RNAs, determining RNA concentrations prior to Northern blot analysis, S1 nuclease or RNase protection assays, cDNA library creation, as well as reverse transcriptase PCR (RT-PCR), and differential display PCR. Traditionally, quantitation of RNA (either total or Poly A+) has been accomplished by measuring light absorbance at 260 nm (A260). While easy to perform, this method has several disadvantages. Free nucleotides and proteins, common contaminants in RNA preparations, can contribute significant amounts of interfering signal when present. DNA cannot be distinguished from RNA with UV light absorbance and must be eliminated enzymatically. In addition, absorbance is relatively insensitive, often requiring large amounts of sample.

Materials and Methods

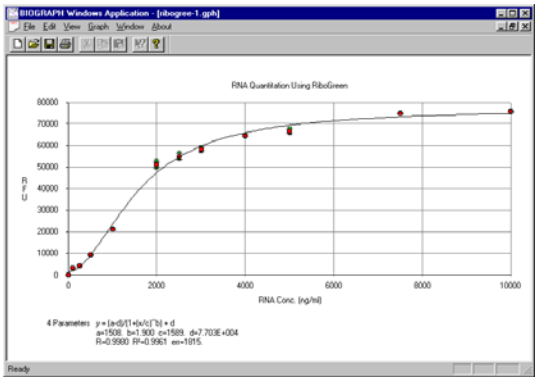
A RiboGreenä RNA quantitation kit, catalogue number O-11492, was purchased from Molecular Probes (Eugene, OR). Black Microfluor B 96-well microplates, catalogue number 7805, were obtained from Dynex (Chantilly, VA). A series of dilutions of total RNA were made using TE buffer (10 mM Tris, 1 mM EDTA pH 7.5) as the diluent. The RNA assay was performed according to the kit instructions (1). For each dilution, 100 µl of each standard was placed in 96-well microplates in replicates of four. To each well, 100 µl of diluted RiboGreen reagent was then added and mixed well. Diluted RiboGreen reagent was prepared fresh by diluting the kit stock solution either 1:200 or 1:2000 with TE buffer as described by the manufacturer. Fluorescence was measured after a 5-minute incubation at room temperature using a FL600 fluorescent plate reader with a 485 nm, 20 nm bandwidth, excitation filter and a 530 nm, 25 nm bandwidth emission filter.

Results

The fluorescence intensity was determined for RNA concentrations ranging from 0 to 10 µg/ml. Within this range four different standard curves were examined. When RNA ranging from 0 to 10 g/ml was stained with a 1:200 dilution of RiboGreen dye a sigmoidal shaped curve is observed (Figure 1A). However, using the same dye concentration, an RNA concentration curve from 0 to 1 µg/ml was observed to be linear (Figure 1B). According to the kit instructions a more diluted fluorescent dye concentration improved detection limits. Using a 1:2000 dilution of the RiboGreen stain, the fluorescence of standard curves ranging from 0 to 100 ng/ml and 0 to 10 ng/ml was determined (Figure 2). For both concentration ranges there is a linear relationship

between fluorescence and RNA. Concentrations as low as 0.5 ng/ml were found to be significantly different from the buffer only sample (Figure 2B). This represents as little as 100 pg of RNA per well.

A



B

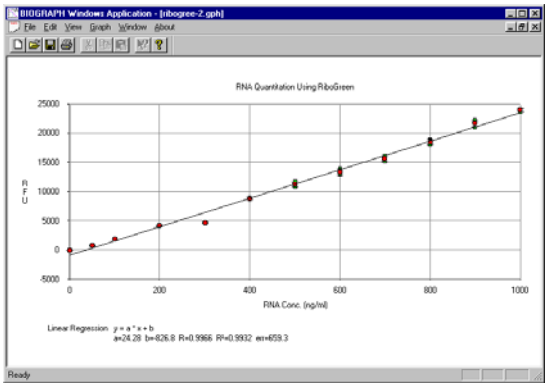
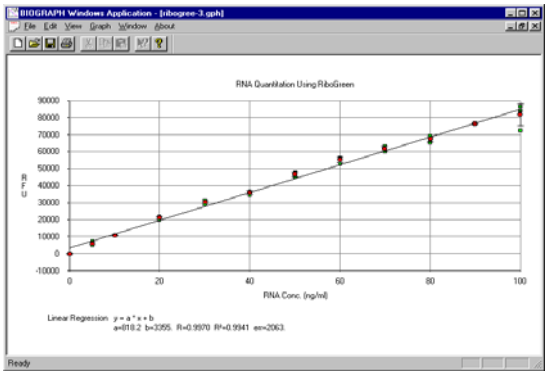


Figure 1. Concentration Curve of High RNA Concentrations Using RiboGreen Stain. (A) The fluorescence of dilutions of total RNA ranging from 0 to 10 µg/ml with a 4-parameter logistic best fit to describe the data. (B) The fluorescence of dilutions of total RNA ranging from 0 to 1 µg/ml with a linear regression of the data. In both figures, the RiboGreen reagent was diluted 1:200 and the fluorescence was determined using a FL600 Fluorescent Plate Reader with a sensitivity setting of 100.

A



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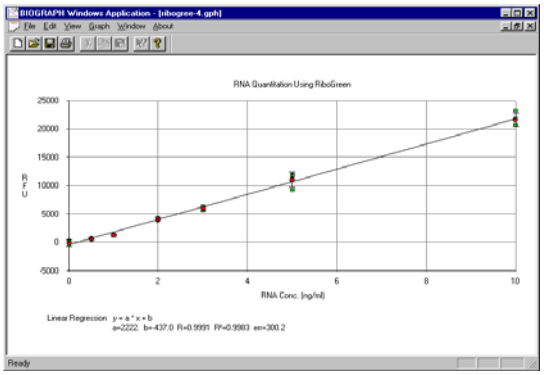

Figure 2. Concentration Curve of low RNA Concentrations Using RiboGreen Stain. The fluorescence of dilutions of total RNA ranging from 0 to 100 ng/ml (A) or 0 to 10 ng/ml (B) was measured. In both figures the RiboGreen reagent was diluted 1:2000 and the fluorescence was determined using a FL600 fluorescent plate reader with a sensitivity setting of 175 for figure A and 200 for figure B. Using KC4 data reduction software the fluorescence of a reagent blank was subtracted from the data and a linear regression was used to describe the data.

Discussion

We have demonstrated that by using RiboGreen stain in conjunction with the FL600 one can detect and quantitate very small amounts of RNA. Although the kit insert claimed a dynamic range from 1 ng to 1 µg/ml (three orders of magnitude), we found that with logistic curve fits concentrations up to 4 µg/ml are easily and reliably quantitated. High concentrations of RNA resulted in a curve that flattened out above 4 µg/ml suggesting that the amount of RiboGreen stain was no longer in excess in relation to the RNA present. One could provide more stain by only diluting the RiboGreen reagent 1:20 in order to increase the upper range, but this quickly becomes expensive. At these concentrations the primary limitation of using absorbance at 260 nm to quantitate nucleic acids, namely its insensitivity, is no longer a problem. Decreasing the stain concentration increased assay sensitivity. By dilution of the stain, the small amount of background fluorescence of the unbound satin was further reduced. This resulted in a lower and more consistent fluorescence of the 0 ng/ml standard.

Although not tested in these experiments, this suggests that lower concentrations may be detected with a further dilution of the stain. Regardless of the dilution, it is critical that the unknown samples and the standards receive the same dilution of fluorescent stain. This fluorescent assay for RNA offers a tremendous increase in sensitivity over existing assays. The use of RiboGreen stain provides a 1000 fold increase in sensitivity over traditional absorbance methods, as well as a 200-fold increase in sensitivity over ethidium bromide assays (2,3). 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 RNA (approximately 100 pg per well) makes it particularly useful. Poly A+ RNA, which is in considerably smaller amounts than ribosomal RNA, can be quantitated without using a large amount of sample. The linearity of the assay is also reported to be maintained in the presence of many contaminants commonly found in RNA preparations, including free nucleotides, urea, proteins, detergents, and agarose.

As with any experiments involving RNA, all solutions and containers should be free of nucleases or contaminating nucleic acids. Clean disposable gloves should be worn during handling and preparation of materials and solutions. In addition, because RiboGreen stain also binds to DNA, treatment of samples with RNase free DNase prior to quantitation is recommended for accurate RNA determination.

References

(1) Molecular Probes, RiboGreen RNA Quantitation Reagent and Kit Instruction, Eugene, Oregon.

(2) LePecq, J.B. and C. Paoletti (1966) A New Fluorometric Method for RNA and DNA Determination, Anal. Biochem. 17:100-107.

(3) Karsten U., and A. Wollenberger (1977) Improvements in the Ethidium Bromide Method for Direct Fluorometric Estimation of DNA and RNA in Cell and Tissue Homogenates, Anal. Biochem. 77:464-470.

(4) Maniatis T., E.F. Fritsch, and J. Sambrook (1982) Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Rev: 9/5/03