A *Pico*fluor™ method for

RNA quantitation using RiboGreen®

1. INTRODUCTION

RiboGreen® RNA Quantitation Reagent is an ultra-sensitive fluorescent nucleic acid stain. It is a simple and rapid procedure for measuring RNA concentration in solution for several molecular biology procedures. A few such procedures include *in vitro* transcription, Northern blot analysis, reverse transcription PCR, differential display PCR, S1 nuclease assays, RNAse protection assays and cDNA synthesis for library production.

The determination of absorbance at 260nm is the most commonly used technique for measuring RNA concentration. Major disadvantages of this method are poor sensitivity (4 µg/ml RNA in solution) and interference in signal levels from contaminating components such as nucleotides, proteins and salts in the RNA solution. The use of an ultra-sensitive fluorescent nucleic acid stain such as RiboGreen® alleviates these problems.

The Turner BioSystems dual-channel *Pico*fluor™ fluorometer along with the Molecular Probes RiboGreen® RNA Quantitation Kit allows rapid and accurate measurement of RNA concentration in solution. The RiboGreen® reagent is non-fluorescent when free in solution. Upon binding RNA, the fluorescence of the RiboGreen® reagent increases more than 1000-fold. The RNA bound RiboGreen® reagent has an excitation maximum of ~500nm and an emission maximum of ~525nm similar to Fluorescein.

The linearity of the assay is maintained even in the presence of contaminants in the RNA solution. RiboGreen® also binds DNA. RNA-DNA mixed samples may be pretreated with DNAse to generate an RNA selective assay. As little as 2.5ng/mL RNA can be quantitated using the blue optical configuration of the *Pico*fluor™. The RiboGreen® assay is ~200-fold more sensitive than ethidium bromide–based assays and ~1000-fold more sensitive than absorbance measurements at 260 nm. The linear dynamic range extends over 4 orders of magnitude from 2.5 ng/mL to 1 µg/mL RNA (Figure 1) using two different concentrations of the RiboGreen® reagent. A linear dynamic range of 10ng/mL to 1µg/mL can be achieved by the use of a high concentration of the dye. Furthermore, by the use of a lower concentration of the dye, a lower range of 2.5ng/mL to 50ng/mL can be measured.

2. MATERIALS REQUIRED

- *Pico*fluor™ Fluorometer with blue optical configuration (P/N 8000-003 or 8000-004).
- 10 mm X 10mm Polystyrene cuvettes (P/N 7000-957).
  We recommend these cuvettes in particular. We found that variations between replicates decreased markedly, leading to better detection limits with these polystyrene cuvettes.
- RiboGreen® RNA Quantitation Kit, (R-11490) Molecular Probes, Inc.
  The kit contains :
  - ❖ 1 mL of RiboGreen® RNA quantitation reagent
  - ❖ 25 mL of 20X TE assay
  - ❖ 5 200 µL aliquots of 100 µg/mL 16S and 23S ribosomal RNA standard (from *E. coli*), in TE buffer.

  Handling, storage and the use of the reagents should be performed in accordance with the product information sheet supplied by Molecular Probes, Inc.
- Nuclease-free water (see Section 3.1, below)

3. EXPERIMENT PROTOCOL

3.1 Assay Buffer Preparation

TE assay buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) is used for diluting the RiboGreen® reagent and the RNA samples. It is imperative that the TE buffer is free of contaminating nucleases and nucleic acids. Clean disposable gloves should be worn during handling and preparation of all materials and solutions. All solutions should be prepared in sterile disposable plastic ware or nuclease-free glassware, using nuclease-free pipettes.

The 20X TE buffer that is included in the RiboGreen® RNA Quantitation Kit is nuclease-free and nucleic acid-free. This buffer is also available from Molecular Probes, Inc. as a separate item (catalog number T-11493). Prepare the 1X TE working solution by diluting the concentrated buffer 20-fold with nuclease-free water.

Nuclease-free water should be prepared by treating distilled, deionized water with 0.1% diethylpyrocarbonate (DEPC), incubating for several hours at 37°C and autoclaving for at least 15 minutes at 15-lbs/sq. inch to sterilize and eliminate DEPC. Caution: DEPC is a suspected carcinogen and should be handled with care. Compounds containing amines, such as Tris, will react rapidly with DEPC and should be added to DEPC treated water only after DEPC is removed by heating. Removal of DEPC by heating is also important to prevent carboxyethylation of the RNA sample. Alternatively, commercially available Nuclease-free water may be used.

3.2 Reagent Preparation

The RiboGreen® RNA Quantitation Reagent is supplied as a 1-mL concentrated dye solution in anhydrous dimethylsulfoxide (DMSO). The DMSO stock solution should be stored at -20°C. Allow the reagent to warm to room temperature.

To measure levels between 1000 –10 ng/mL RNA, prepare the high range assay reagent by making a 1:200 dilution of the concentrated RiboGreen® Reagent into TE. A dilution of 100µL RiboGreen® Quantitation Reagent to 20.0 mL TE is an adequate working solution to assay 20 samples in 2mL volumes.

To measure levels between 50 –2.5 ng/mL RNA, prepare the low range assay reagent by making a 1:2000 dilution of the concentrated RiboGreen® Reagent into TE. A dilution of 10µL RiboGreen® Quantitation Reagent to 20.0 mL TE is an adequate working solution to assay 20 samples in 2mL volumes.

Prepare these solutions in a plastic container, as the reagent may adsorb to glass surfaces. The working solution of the RiboGreen® Reagent must be protected from light with foil or placing it in the dark to prevent photo degradation. **For best results, this solution should be used within a few hours of its preparation.**

3.3 RNA Standard Curve

1. Prepare a 2µg/mL solution of RNA in TE using nuclease-free plastic ware. Determine the RNA concentration on the basis of absorbance at 260 nm (A260) in a cuvette with a 1 cm pathlength; an A260 of 0.05 corresponds to 2 µg/mL RNA.

2. The 16S and 23S ribosomal RNA standard, provided at 100 µg/mL in the RiboGreen® RNA Quantitation Kit, can simply be diluted 50-fold in TE to make the 2 µg/mL working solution. For example, 40 µL of the RNA standard mixed with 1.96 mL of TE will be sufficient for the standard curve described below in Table 1.

3. For the high-range standard curve, dilute the 2 µg/mL RNA solution into disposable cuvettes as shown in Table 1. For the low-range standard curve, first dilute the 2 µg/mL RNA solution 20-fold with TE buffer to make a 100 ng/mL RNA stock solution and use this to prepare the dilutions shown in Table 2.

4. Add 1.0 mL of the appropriate aqueous working solution of RiboGreen® reagent (prepared in Section 3.2) to each cuvette. The high-range working reagent should only be used for performing the high-range assay and the low-range working reagent should only be used for performing the low-range assay. Mix well and incubate for 2 to 5 minutes at room temperature, protected from light.

5. Set-up the *Pico*fluor™ fluorometer as per instructions in the User's manual. Power up the instrument by pressing the [ON/OFF] button. Use the [A/B] button to toggle to the "Blue"

channel. Press [STD VAL] to program in the concentration of your calibration standard. Use the up and down arrows to change the concentration value. Hold down the arrow key to activate faster scrolling. When ready, press the [CAL] button to start the calibration. The *Pico*fluor's screens will lead you through the calibration process.

6. Measure the fluorescence of the remaining standards to generate a standard curve of fluorescence versus RNA concentration.

Table 1. Protocol for preparing high range standard curve.

Vol.(uL) 2ug/mL RNA stock	Vol. (uL) of TE	Vol. (uL) diluted RiboGreen® reagent	Final RNA concentration in RiboGreen® assay
1000	0	1000	1000 (ng/mL)
750	250	1000	750 (ng/mL)
500	500	1000	500 (ng/mL)
250	750	1000	250 (ng/mL)
100	900	1000	100(ng/mL)
50	950	1000	50 (ng/mL)
25	975	1000	25 (ng/mL)
10	990	1000	10 (ng/mL)
0	1000	1000	0 (ng/mL)

Table 2. Protocol for preparing low-range standard curve.

Vol. (uL) of 100ng/mL RNA stock	Vol. (uL) of TE	Vol. (uL) Diluted RiboGreen® reagent	Final RNA concentration in RiboGreen® assay
1000	0	1000	50 (ng/mL)
600	400	1000	30 (ng/mL)
500	500	1000	25 (ng/mL)
400	600	1000	20 (ng/mL)
300	700	1000	15 (ng/mL)
200	800	1000	10 (ng/mL)
100	900	1000	5 (ng/mL)
50	950	1000	2.5 (ng/mL)
20	980	1000	1 (ng/mL)
0	1000	1000	0 (ng/mL)

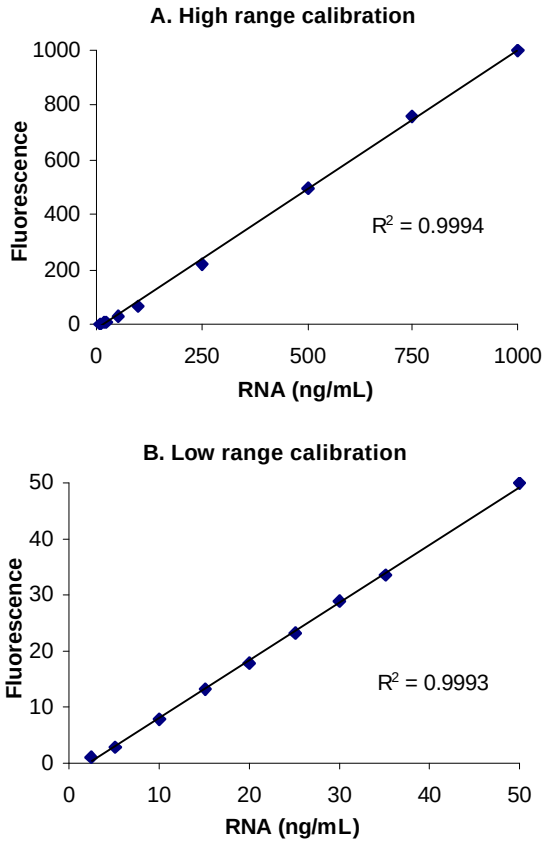
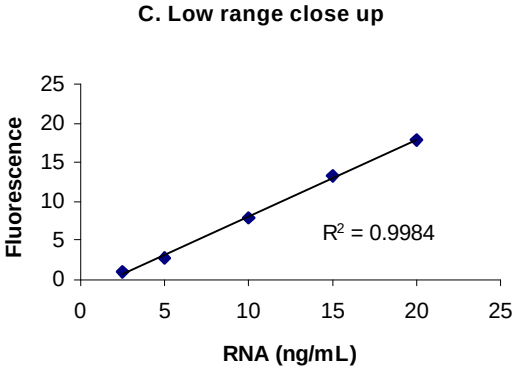


Figure 1. High range (A), low range (B) and low range close up (C) *E. coli* ribosomal



RNA standard assays performed using RiboGreen® RNA quantitation reagent and the *Pico*fluor™ handheld fluorometer. Sensitivity calibrations were performed separately for the high and low assay ranges.

3.4 Sample Analysis

1. Dilute each experimental RNA solution in TE to a final volume of 1.0 mL in disposable cuvettes or test tubes. It may be useful to prepare several dilutions of each experimental sample. For example, if a series of RNA samples contain widely differing salt concentrations, they cannot be compared to a single standard curve. To avoid this problem, simply adjust the concentration of contaminants to be the same in all samples, if possible. See Section 3.5 for information on eliminating DNA from the sample.
2. Add 1.0 mL of the appropriate RiboGreen® reagent (prepared in Section 3.2) to each sample. Incubate for 2 to 5 minutes at room temperature, protected from light.
3. Measure the fluorescence of each sample using the same instrument calibration conditions as used to generate the standard curve (see Section 3.3).
4. If the standard curve has been constructed from background-subtracted data (see Section 3.3), subtract the reagent blank fluorescence reading from that of each of the samples.
5. Determine the RNA concentration of each sample from the standard curve you generated in Section 3.3.

3.5 Eliminating DNA from Samples

RiboGreen® reagent also binds to DNA. The following procedure for pre-treating the sample with RNase-free DNase can eliminate this background fluorescence:

1. Prepare 10X DNase digestion buffer: nuclease-free 200 mM Tris-HCl, pH 7.5, containing 100 mM $MgCl_2$ and 20 mM $CaCl_2$.
2. Add 0.11 sample volume of 10X DNase digestion buffer to each DNA-containing sample (for example, to a 9 µL sample, add 1 µL 10X buffer).
3. Add about 5 units of RNase-free DNase I per µg of DNA thought to be in the sample.
4. Incubate the sample at 37°C for 90 minutes.
5. Dilute the sample 10X in TE to keep the effects of the digestion buffer salts to a minimum.
6. Perform the RiboGreen® assay.

4. REFERENCES

1. Anal. Biochem. 17, 100 (1966).
2. Anal. Biochem. 265, 368 (1998).
3. Molecular Cloning: A Laboratory Manual, Second Edition, J. Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

5. WARNINGS AND PRECAUTIONS

The RiboGreen® RNA Quantitation Reagent is the subject of patent applications filed by Molecular Probes, Inc. and is not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. RiboGreen® is a registered trademark of Molecular Probes, Inc.

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