A Modulus™ Microplate Fluorometer Method for

RNA Quantitation Using Quant-iT™ RiboGreen® RNA Assay

1. INTRODUCTION

Quant-iT™ RiboGreen® RNA Quantitation Reagent is an ultrasensitive fluorescent nucleic acid stain. Use of this reagent allows for simple and rapid measuring of RNA concentration in solution for a multitude of 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 most commonly used technique for measuring RNA concentration is the determination of absorbance at 260 nm. 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 Modulus™ Microplate Fluorometer used in conjunction with the Invitrogen RiboGreen® RNA Assay Kit allows for rapid and accurate measurement of RNA concentrations in small-volume microplates (200 µL per well). The RiboGreen® reagent is non-fluorescent when free in solution; upon binding to RNA, the fluorescence of the RiboGreen® reagent increases more than 1000-fold. The RNA-bound RiboGreen® reagent has an excitation maximum of approximately 500 nm and an emission maximum of approximately 525 nm.

As little as 50 pg of RNA can be quantitated using the Modulus™ Microplate Fluorometer Blue Fluorescence Optical Kit. The Quant-iT™ RiboGreen® RNA Assay is approximately 200-fold more sensitive than ethidium bromide-based assays and approximately 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 - 1 µg/mL RNA (Figure 1) using two different concentrations of the RiboGreen® reagent. A linear dynamic range of 10 ng/mL - 1 µg/mL can be achieved by using high dye concentration. Furthermore, by using a low dye concentration, a lower range of 1 ng/mL – 50 ng/mL can be measured.

2. MATERIALS REQUIRED

- Modulus™ Microplate Multimode Reader
- Fluorescence Optical Kit - Blue, 490/515-580 nm
- Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, R11490), containing:
  - Quant-iT™ RiboGreen® RNA reagent (1 mL solution in DMSO)
  - 20 x TE (25 mL in DEPC-treated water)
  - 16S and 23S ribosomal RNA standards from *E. coli* (5 aliquots each containing 200 µL of 100 µg/mL in TE)

  NOTE: Handling, storage and the use of the reagents should be performed in accordance with the product information sheet supplied by Invitrogen.
- Nuclease-free water (Invitrogen, T11493)
- Black 96-well microplates FluoTrac 200 (E&K Scientific, EK-25076)

3. EXPERIMENTAL 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 RNA samples. The TE buffer must be free of all 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 plasticware or nuclease-free glassware, using nuclease-free pipettes.

The Quant-iT™ RiboGreen® RNA Quantitation Kit includes 20 x TE buffer which is free of nuclease and nucleic acid. This buffer is also available from Molecular Probes, Inc. as a separate item (T-11493).

Prepare the 1 x TE working solution by diluting the concentrated buffer 20-fold with nuclease-free water.

Nuclease-free water should either be purchased or 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/inch^2 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 the DEPC is removed by heating. Removal of DEPC by heating is also important to prevent carboxyethylation of the RNA sample.

3.2 Reagent Preparation

The RiboGreen® RNA Quantitation Reagent is supplied as a 1-mL solution of concentrated dye in anhydrous dimethylsulfoxide (DMSO). Allow the reagent to warm to room temperature.

To measure levels of 1000 - 10 ng/mL RNA, prepare the high-range assay reagent by making a 1:200 dilution of concentrated RiboGreen® Reagent into TE. A dilution of 100 µL RiboGreen® Quantitation Reagent to 20.0 mL TE is an adequate working solution to assay 20 samples in 2-mL volumes. To measure levels of 50 - 2.5 ng/mL RNA, prepare the low-range assay reagent by making a 1:2000 dilution of concentrated RiboGreen® Reagent into TE. A dilution of 10 µL RiboGreen® Quantitation Reagent to 20 mL TE creates adequate working solution to assay 20 samples in 2-mL volumes.

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

3.3 RNA Standard Curve

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

2. For the high-range standard curve, dilute the 2-µg/mL RNA solution 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. Add 100 µL of each standard to separate wells of a 96-well assay plate. It is recommended to perform duplicates or triplicates of each standard to determine an accurate standard curve.

3. Add 100 µL of the appropriate aqueous working solution of RiboGreen® reagent (prepared in Section 3.2) to each well. The high-range working reagent should only be used for performing the high-range assay; low-range working reagent should only be used for performing the low-range assay. Mix well and incubate for 2 - 5 minutes at room temperature, protected from light.

4. Set up the Modulus™ Microplate Fluorometer as per instructions in the *Operating Manual* and read the assay plate.

5. Subtract the fluorescence value of the reagent blank from each sample. Use corrected data to

generate a standard curve of fluorescence verses RNA concentration.

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

Table 1. Protocol for Preparing High-Range Standard Curve

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

Table 2. Protocol for Preparing Low-Range Standard Curve

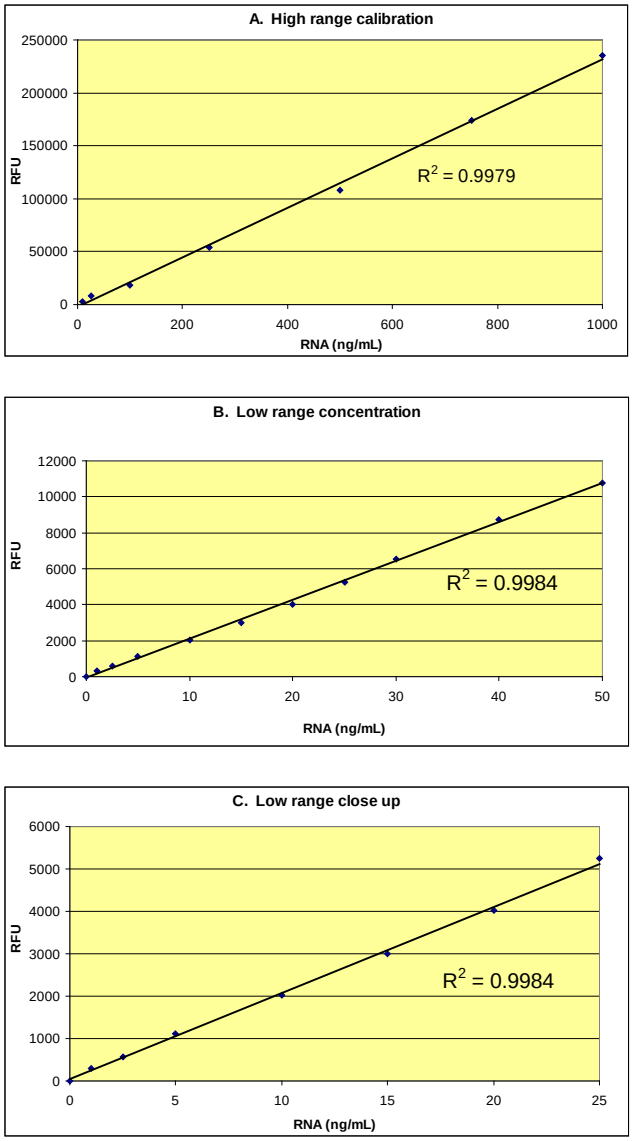

Figure 1A - C. *E. coli* ribosomal RNA standard assays performed using RiboGreen® RNA quantitation reagent and the Modulus™ Microplate Fluorometer. Sensitivity calibrations were performed separately for the high- and low-assay ranges.

3.4 Sample Analysis

1. Dilute each experimental RNA solution in TE to a final volume of 100 uL and add to a microtiter plate. 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 100 uL of the appropriate RiboGreen® reagent (prepared in Section 3.2) to each sample. Incubate 2 - 5 minutes at room temperature, protected from light.

3. Measure the fluorescence within samples by using the same instrument conditions as were 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's blank fluorescence reading from that of each sample.

5. Determine the RNA concentration of each sample from the standard curve generated in Section 3.3.

3.5 Eliminating DNA from Samples

Since RiboGreen® also binds DNA, RNA-DNA mixed samples may be pretreated with DNase to generate an RNA-selective assay. The following procedure for pre-treating the sample with RNase-free DNase can eliminate background fluorescence.

1. Prepare 10 x 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 10 x DNase digestion buffer to each DNA-containing sample (for example, to a 9-mL sample, add 1 mL of 10 x buffer).

3. Add approximately 5 units of RNase-free DNase I per µg of DNA in the sample.

4. Incubate the sample at 37° C for 90 minutes.

5. Dilute the sample 10 x in TE to keep the effects of digestion buffer salts to a minimum.

6. Perform the Quant-iT™ 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. RESULTS

Sensitivity: < 1 ng/mL

Instrument Dynamic Range: Up to 6 orders of magnitude within dynamic range

Minimum Detection Limit: 250 pg/mL, Calculated using three times standard deviation of the assay background, n = 24

6. CONCLUSION

The Modulus™ Microplate Fluorometer offers both superior sensitivity and dynamic range. The Modulus™ Microplate Fluorometer achieves its superior performance by use of a dedicated fluorescence detector instead of sharing equipment with other detection modes. The individual Optical Kit offers solid-state optics and a powerful wavelength-matched LED to deliver excellent sensitivity and dynamic range.

The modular approach of the Modulus™ Microplate Fluorometer allows for instrument capability expansion as needs in the lab change. Luminescence and/or Absorbance Detection Modules as well as other accessories can be added after the initial purchase.

The superior performance, ease of use, and utmost flexibility of the Modulus™ Microplate makes it an ideal microplate reader for today's life science laboratory.

7. WARNINGS AND PRECAUTIONS

Care should be taken to prevent RNase contamination of the Quant-iT™ RiboGreen® reagent and kit components. Clean disposable gloves should be worn while handling all materials.

Caution: No data are available addressing the mutagenicity or toxicity of Quant-iT™ RiboGreen® RNA reagent.

Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solution. As with all nucleic acid reagents, solutions of Quant-iT™ RiboGreen® reagent should be poured through activated charcoal before disposal. Incinerate the contaminated charcoal to destroy the dye.

TRADEMARKS

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