A TBS-380 Mini-Fluorometer Method for RNA Quantitation Using RiboGreen®

1. INTRODUCTION

The Turner BioSystems TBS-380 Mini-Fluorometer in combination with Molecular Probes' RiboGreen® RNA quantitation reagent provides a method for ultrasensitive quantitation of RNA in solution. Detecting and quantitating small amounts of RNA is extremely important for a wide variety of molecular biology procedures. These include measuring yields of *in vitro* transcribed RNA, measuring RNA concentrations before performing Northern blot analysis, S1 nuclease assays, RNase protection assays, cDNA library preparation, and reverse transcription PCR and differential display PCR.

The conventional technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A_{260}). The major disadvantages of the A_{260} method are the relative large contribution of proteins and free nucleotides to the signal, the inability to distinguish between DNA and RNA, the interference caused by contaminants commonly found in nucleic acid preparations, and the relative insensitivity of the assay (an A_{260} of 0.1 corresponds to a 4 µg/mL RNA solution). The use of fluorescent nucleic acid stains alleviates many of these problems.

The RiboGreen® RNA quantitation assay used in conjunction with the TBS-380 Mini-Fluorometer can detect as little as 1 ng/mL RNA (Figure 1), exceeds the sensitivity of ethidium bromide–based fluorometric assays[1] by 200-fold and A_{260} measurements by 1000-fold. The linear quantitation range extends over two and half orders of magnitude in RNA concentration, using two dye concentrations. Using one concentration of RiboGreen® reagent and the recommended assay protocol, researchers can quantitate 25 ng/mL to 500 ng/mL RNA. By diluting the RiboGreen® reagent 10-fold further, 1 ng/mL to 50 ng/mL RNA can be quantitated. Linearity is maintained in the presence of several compounds commonly found to contaminate nucleic acid preparations. Although the RiboGreen® reagent also binds to DNA, pretreatment of mixed samples with DNase can be used to generate an RNA-selective assay.

2. MATERIALS REQUIRED

- TBS-380 Mini-Fluorometer (P/N 3800-003).
- 10×10 mm square methacrylate cuvettes (P/N 7000-959).
- Minicell Adaptor Kit (P/N 3800-928).
- RiboGreen® RNA Quantitation Kit, supplied by Molecular Probes, Inc., Eugene, OR (catalog number R-11490). The kit contains 1 mL of RiboGreen® RNA quantitation reagent stock solution in DMSO, 25 mL of 20X TE assay buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5 in DEPC (diethylpyrocarbonate–treated water) and 1 mL (supplied as 5 x 200 µL) of 100 µg/mL 16S and 23S ribosomal RNA standard (from *E. coli*), in TE buffer. The kit contents are sufficient for 200 high-range (20 ng/mL to 1 µg/mL) RNA assays using 2.0 mL samples in 10 mm × 10 mm cuvettes. RiboGreen® RNA Quantitation Reagent (1 mL in DMSO) is also available from Molecular Probes as a separate item (catalog number R-11491). 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 3.2, below).

3. EXPERIMENTAL PROTOCOL

3.1 Overview

Two different dye concentrations are required to achieve the full linear dynamic range of the RiboGreen® RNA quantitation assay. Different working solutions of RiboGreen® reagent are prepared for the **high-range** assay (25 ng/mL to 500 ng/mL RNA) and the **low-range** assay (1 ng/mL to 50 ng/mL RNA), as described below in Section 3.3.

3.2 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 plasticware 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 PSI 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.[2]

3.3 Reagent Preparation

On the day of the experiment, prepare a 2X working solution of the RiboGreen® reagent by diluting an aliquot of the concentrated DMSO stock solution into 1X TE. If performing the **high-range** assay, dilute 200-fold. For example, to prepare enough working solution to assay 20 samples in 2 mL volumes, add 100 µL RiboGreen® RNA quantitation reagent to 19.9 mL 1X TE. If performing the **low-range** assay, dilute 2000-fold. For example, to prepare enough working solution to assay 20 samples in 2 mL volumes, add 10 µL RiboGreen® RNA quantitation reagent to 20.0 mL 1X TE. Prepare these solutions in sterile, disposable, polypropylene plasticware rather than glassware, as the reagent may adsorb to glass surfaces. Protect the working solutions from light by covering them with foil or placing them in the dark, as the RiboGreen® reagent is susceptible to photodegradation.
For best results, these solutions should be used within a few hours of their preparation.

3.4 RNA Standard Curves

3.4.1 Prepare a 2 µg/mL solution of RNA in 1X TE using nuclease-free plasticware. Determine the RNA concentration on the basis of absorbance at 260 nm (A_{260}) in a cuvette with a 1 cm pathlength; an A_{260} of 0.05 corresponds to 2 µg/mL RNA. 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 1XTE 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. It is sometimes preferable to prepare the standard curve with purified RNA similar to the type being assayed. In general, equivalent amounts of single-stranded RNA from different sources produce approximately equal fluorescence intensity readings. The assay remains linear in the presence of several compounds that commonly contaminate nucleic acid preparations, including nucleotides, salts, urea, ethanol, chloroform, detergents, proteins and agarose. However the fluorescence intensity may be affected (see Molecular Probes product information sheet MP11490 for details) and therefore the RNA solution used to prepare the standard curve should be treated the same way as the experimental samples and should contain similar levels of such compounds.

3.4.2 For the **high-range** standard curve, make a series of RNA standard solution at 2X final concentration by diluting the 2 µg/mL RNA solution into disposable cuvettes or nuclease-free plastic test tubes for transfer to Minicell cuvettes (see table 1).

2X RNA solution concentration (ng/mL)	Volume of the 2X RNA solution (mL)	Volume of the 2X high-range working solution (mL)	Final RNA Concentration in RiboGreen® Assay (ng/mL)
1000	1	1	500
200	1	1	100
100	1	1	50
50	1	1	25
0	1	1	blank

Table 1. *High-range RNA standard curve for* ***10x10 mm cuvette.***

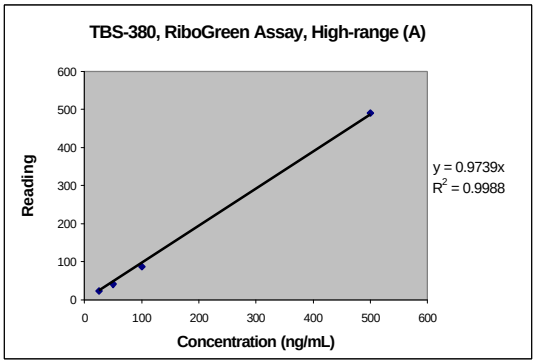
For the **low-range** standard curve, make a series of RNA standard solution at 2X final concentration by diluting the 2 µg/mL RNA solution into disposable cuvettes or nuclease-free plastic test tubes for transfer to Minicell cuvettes (see Table 2).

2X RNA solution concentration (ng/mL)	Volume of the 2X RNA solution (mL)	Volume of the 2X low-range working solution (mL)	Final DNA concentration in RiboGreen Assay (ng/mL)
100	1	1	50
50	1	1	25
20	1	1	10
10	1	1	5
2	1	1	1
0	1	1	blank

Table 2. *Low-range RNA standard curve for* ***10x10 mm cuvette****.*

3.4.3 Mix equal volume of the 2X working solution of RiboGreen® reagent (prepared in Section 3.3) with each 2X RNA standard solution. The **high-range** working solution (200-fold dilution of stock) should only be used for performing the **high-range** assay and the **low-range** working solution (2000-fold dilution of stock) 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. Be sure to add enough volume into the cuvettes. The minimum volume is 2 mL for 10x10 mm cuvette and 50 µL for Minicell cuvette.

3.4.4 Select the BLUE channel of the TBS-380 Mini-Fluorometer. Calibrate the fluorometer using the sample containing the highest concentration of RNA [Note: For optimal detection sensitivity, separate calibrations should be carried out for the high range and low range assays]. Measure the fluorescence of the remaining samples. The TBS-380 Mini-Fluorometer will give a direct concentration read out, and data may be used to generate a standard curve of reading versus RNA concentration.



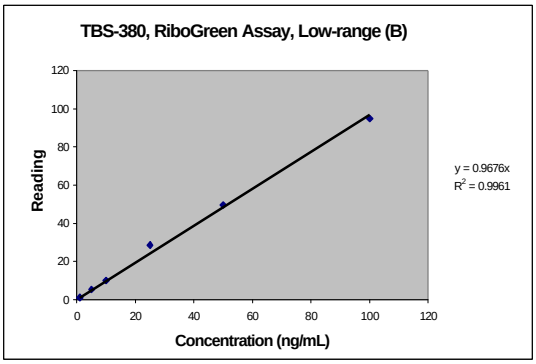


Figure 1. High-range (A) and low-range (B) ribosomal RNA standard assays performed using RiboGreen® RNA Quantitation Reagent and the TBS-380 Mini-Fluorometer. Separate instrument calibrations were carried out for the two assay ranges.

3.5 Sample Analysis

3.5.1 Dilute each unknown RNA samples in 1X TE to a desired volume (1.0 mL for 10x10 mm cuvette or 25-100 µL for Minicell). It may be useful to prepare several dilutions of each experimental sample. Large dilutions of the experimental sample may serve to diminish the interfering effect of certain contaminants. However, extremely small sample volumes should be avoided because they are difficult to pipet accurately. In addition, the level of assay contaminants should be kept as uniform as possible throughout an experiment, to minimize sample-to-sample signal variation. 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 3.6 for information on eliminating DNA from the sample.

3.5.2 Add equal volume of the 2X working solution of the RiboGreen® reagent (prepared in Section 3.3) to each sample. Incubate for 2 to 5 minutes at room temperature, protected from light.

3.5.3 Measure the fluorescence of each sample using the same instrument calibration conditions as used to generate the standard curve (see 3.4.4).

3.6 Eliminating DNA from Samples

RiboGreen® reagent also binds to DNA. Fluorescence in samples that is due to RiboGreen® reagent binding to DNA can be eliminated by pre-treating the sample with RNase-free DNase, ensuring that the entire sample fluorescence is due to dye bound to RNA.

3.6.1 Prepare 10X DNase digestion buffer: nuclease-free 200 mM Tris-HCl, pH 7.5, containing 100 mM $MgCl_2$ and 20 mM $CaCl_2$.

3.6.2 Add 0.11 sample volume of 10X DNase digestion buffer to each DNA-containing sample (for example, to a 9 mL sample, add 1 mL 10X buffer).

3.6.3 Add about 5 units of RNase-free DNase I per mg of DNA thought to be in the sample.

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

3.6.5 Dilute the sample at least 10-fold into TE to diminish effects of the digestion buffer salts on the RiboGreen® assay procedure.

3.6.6 Perform the RiboGreen® assay as described above.

4. REFERENCES

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

5. PATENT & TRADEMARK INFORMATION

RiboGreen is a registered trademark of Molecular Probes, Inc. RiboGreen® RNA Quantitation Reagent is covered by current or pending U.S. and foreign patents.

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