Quant-iT™ RiboGreen® RNA Reagent and Kit

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
Quant-iT™ RiboGreen® RNA Reagent (Component A) (R11491, R11490)	1 mL	Solution in DMSO	• 2–6°C • Desiccate • Protect from light	When stored as directed, products are stable for at least 1 year. *For long-term storage, store the rRNA standards at ≤–20°C or –70°C.
20X TE Buffer, RNase-free (Component B) (R11490)	25 mL	200 mM Tris-HCl, 20 mM EDTA, pH 7.5 in DEPC-treated water	• ≤25°C	
Ribosomal RNA standard, 16S and 23S rRNA from *E. coli* (Component C) (R11490)	5 × 200 µL	100 µg/mL in TE buffer	• 2–6°C* • Avoid freeze-thaw cycles	
Number of assays: 200–2,000 cuvette assays using a 2 mL sample volume or 2,000–20,000 microplate assays using a 200 µL sample volume.				
Approximate fluorescence excitation/emission maxima: 500/525 nm, bound to nucleic acid.				

Introduction

Quant-iT™ RiboGreen® RNA reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating RNA in solution. Invitrogen offers the Quant-iT™ RiboGreen® reagent as a stand-alone reagent (Cat. no. R11491) or as part of a convenient kit (Cat. no. R11490), which also includes concentrated assay buffer and a ribosomal RNA standard. 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 and measuring RNA concentrations before performing Northern blot analysis, S1 nuclease assays, RNase protection assays, cDNA library preparation, reverse transcription PCR, and differential display PCR.

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A_{260}). The major disadvantages of the absorbance-based method are the large relative contribution of proteins and free nucleotides to the signal, 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 sensitive, fluorescent nucleic acid stains alleviates many of these problems.

The Quant-iT™ RiboGreen® RNA reagent enables quantitation of as little as 1 ng/mL RNA with a fluorescence microplate reader, standard spectrofluorometer, or filter fluorometer, using fluorescein excitation and emission wavelengths.[1] The excitation maximum for Quant-

iT™ RiboGreen® reagent bound to RNA is ~500 nm and the emission maximum is ~525 nm. Using a fluorescence microplate reader, as little as 200 pg RNA can be detected in a 200 µL assay volume.[1] This sensitivity exceeds that achieved with ethidium bromide–based assays [2] by 200-fold and exceeds that achieved with ultraviolet absorbance determination by 1,000-fold. The linear range for quantitation with Quant-iT™ RiboGreen® reagent extends over three orders of magnitude in RNA concentration—from 1 ng/mL to 1 µg/mL RNA—using two dye concentrations [1] (Figure 1). The high-range assay allows quantitation of 20 ng/mL to 1 µg/mL RNA, and the low-range assay allows quantitation of 1 ng/mL to 50 ng/mL RNA. This linearity is maintained in the presence of several compounds commonly found to contaminate nucleic acid preparations, including nucleotides, salts, urea, ethanol, chloroform, detergents, proteins, and agarose.[1] In addition, although the Quant-iT™ RiboGreen® reagent also binds to DNA, pretreatment of mixed samples with DNase can be used to generate an RNA-selective assay[1] (see *Eliminating DNA from Samples* at the end of this document).

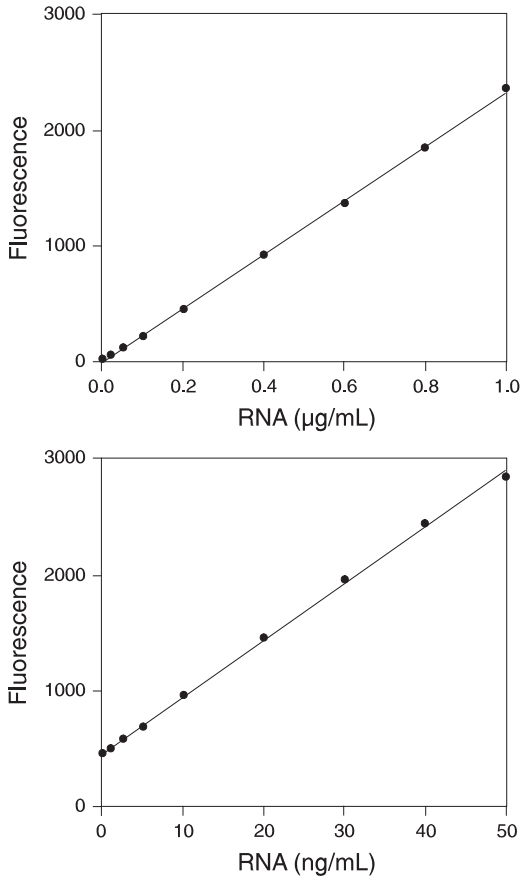

Figure 1. Dynamic range and sensitivity of the Quant-iT™ RiboGreen® RNA assay. For the high-range assay (top panel), Quant-iT™ RiboGreen® reagent was diluted 200-fold into 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE) and 100 µL of the reagent solution was added to microplate wells containing 100 µL ribosomal RNA in TE. For the low-range assay (bottom panel), Quant-iT™ RiboGreen® reagent was diluted 2,000-fold into TE, and 100 µL of the reagent solution was added to microplate wells containing 100 µL of ribosomal RNA in TE. Samples were excited at 485 ± 10 nm and the fluorescence emission intensity was measured at 530 ± 12.5 nm using a fluorescence microplate reader. Fluorescence emission intensity was then plotted versus RNA concentration.

Before You Begin

Handling and Disposal

Prevent RNase contamination of the Quant-iT™ RiboGreen® reagent and kit components. Always use clean disposable gloves 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, treat the reagent as a potential mutagen and handle with appropriate care. Handle the DMSO stock solution 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, pour solutions of Quant-iT™ RiboGreen® reagent through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dye.

Preparing Assay Buffer

TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) is used for diluting the Quant-iT™ RiboGreen® reagent and for diluting RNA samples. Make sure the TE solution is free of contaminating nucleases and nucleic acids. Wear clean disposable gloves during handling and preparation of all materials and solutions. Prepare all solutions in sterile disposable plasticware or nuclease-free glassware, using nuclease-free pipettes.

The 20X TE buffer included in the Quant-iT™ RiboGreen® RNA Assay Kit is nuclease-free and nucleic acid–free. Prepare the 1X TE working solution by diluting the concentrated buffer (Component B) 20-fold with nuclease-free water. Prepare nuclease-free water by treating distilled, deionized water with 0.1% diethyl pyrocarbonate (DEPC), incubating for several hours at 37°C, and autoclaving for at least 15 minutes at 15 lbs/sq. inch to sterilize the water and eliminate DEPC.

Caution: DEPC is a suspected carcinogen and should be handled with care. Compounds containing amines, such as Tris, 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]

Materials Required but Not Provided

- Nuclease-free water
- Sterile, disposable polypropylene plasticware for reagent preparation
- Fluorescence cuvettes or 96-well plates for microplate assay

Experimental Protocols

Overview

Two different dye concentrations are required to achieve the full linear dynamic range of the Quant-iT™ RiboGreen® RNA assay. Before preparing the working solution of Quant-iT™ RiboGreen® reagent (see **Preparing Reagent** below), decide whether you wish to perform the **high-range** assay (20 ng/mL to 1 µg/mL RNA), **low-range** assay (1 ng/mL to 50 ng/mL RNA), or both. Described below are protocols for performing both procedures. In addition, the procedure below has 2 mL assay volumes and is designed for use with standard fluorescence cuvettes. To perform microplate assays, reduce the indicated volumes appropriately. For example, 200 µL assay volumes are recommended for use with 96-well microplates.

Preparing Reagent

On the day of the experiment, prepare an aqueous working solution of the Quant-iT™ RiboGreen® reagent as described below in sterile, disposable polypropylene plasticware rather

than glassware, as the reagent may adsorb to glass surfaces. Protect the working solution from light by covering with foil or placing it in the dark, as the Quant-iT™ RiboGreen® reagent is susceptible to photodegradation. **For best results, use the working solution within a few hours of their preparation.**

Note: Allow the Quant-iT™ RiboGreen® reagent to warm to room temperature before opening the vial. Cold DMSO solutions absorb moisture from warmer, room temperature air, resulting in loss of efficacy for the reagent. Always store the DMSO stock solution in the presence of desiccant when not in use.

Prepare the aqueous Quant-iT™ RiboGreen® reagent working solution by diluting an aliquot of the concentrated DMSO stock solution (Component A) into TE, 200-fold for the **high-range** assay or 2,000-fold for the **low-range** assay. For a 2-mL cuvette assay, you need 1 mL of the Quant-iT™ RiboGreen® RNA reagent working solution per sample. For microplate assays of a total 200 µL assay volume, you need 100 µL of the Quant-iT™ RiboGreen® RNA reagent working solution per sample.

For example, to prepare enough working solution to assay 20 samples in 2 mL volumes, add 100 µL Quant-iT™ RiboGreen® RNA reagent to 19.9 mL TE for **high-range** assay or to prepare enough working solution to assay 20 samples in 2 mL volumes, add 10 µL Quant-iT™ RiboGreen® RNA reagent to 20.0 mL TE for **low-range** assay.

RNA Standard Curves

For a standard curve, we commonly use 16S and 23S ribosomal RNA, although any purified RNA preparation may be used. It is sometimes preferable to prepare the standard curve with RNA similar to the type being assayed. However, we have found that most single-stranded RNA molecules yield approximately equivalent signals. Our results have shown that the assay remains linear in the presence of several compounds that commonly contaminate nucleic acid preparations, although the signal intensity may be affected (Table 1). Thus, to serve as an effective control, 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.

1.1 Prepare a 2 µg/mL solution of RNA in 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 ribosomal RNA standard (Component C), provided at 100 µg/mL in the Quant-iT™ RiboGreen® Assay Kit (R11490) is 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 is sufficient for the standard curve described below.

1.2 For the high-range standard curve, dilute the 2 µg/mL RNA solution into disposable cuvettes (or nuclease-free plastic test tubes for transfer to quartz cuvettes or microplates) as shown in Table 2. For the low-range standard curve, dilute the 2 µg/mL RNA solution 20-fold into TE to make a 100 ng/mL RNA stock solution, and then prepare the dilution series shown in Table 3.

1.3 Add 1.0 mL of the appropriate aqueous working solution of Quant-iT™ RiboGreen® reagent (prepared above) to each cuvette. Use the high-range working solution for performing the high-range assay, and use the low-range working solution or performing the low-range assay. Mix well and incubate for 2 to 5 minutes at room temperature, protected from light.

1.4 Measure the sample fluorescence using a spectrofluorometer or fluorescence microplate reader and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm).

To ensure that the sample readings remain in the detection range of the fluorometer, set the instrument's gain so that the sample containing the highest RNA concentration yields a fluorescence intensity near the fluorometer's maximum. For optimal detection sensitivity, the instrument gain may be increased for the low-range assay relative to the high-range assay. To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.

Table 1. Effects of several compounds that commonly contaminate nucleic acid preparations on the signal intensity of the Quant-iT™ RiboGreen® RNA assay.

Compound	Maximum Acceptable Concentration	% Signal Change*
Salts		
Ammonium acetate	20 mM	4% decrease
Sodium acetate	20 mM	11% decrease
Sodium chloride	20 mM	15% decrease
Zinc chloride	1 mM	9% decrease
Magnesium chloride	0.5 mM	9% decrease
Calcium chloride	0.1 mM	2% increase
Cesium chloride	10 mM	8% decrease
Guanidinium thiocyanate	10 mM	9% decrease
Urea	3 M	13% decrease
Organic Solvents		
Phenol	0.5%	5% decrease
Ethanol	20%	10% decrease
Chloroform	2%	15% increase
Detergents		
Sodium dodecyl sulfate	0.05%	10% decrease
Triton® X-100	0.5%	8% decrease
Proteins		
Bovine serum albumin	0.2%	11% decrease
IgG	0.02%	4% decrease
Other Compounds		
Formamide	10%	12% decrease
Sucrose	>500 mM	4% decrease
Boric acid	100 mM	15% decrease
Polyethylene glycol	10%	10% decrease
Agarose	0.2%	3% increase

*The compounds were incubated at the indicated concentrations with Quant-iT™ Ribogreen® reagent in the presence of 1.0 mg/mL ribosomal RNA. All samples were assayed in a final volume of 200 µL in 96-well microplates using a fluorescence microplate reader. Samples were excited at 485 ± 10 nm and fluorescence intensity was measured at 530 ± 12.5 nm.

Table 2. Protocol for preparing a high-range standard curve.

Volume (µL) of TE	Volume (µL) of 2 µg/mL RNA Stock	Volume (µL) of 200-fold Diluted Quant-iT™ RiboGreen® Reagent	Final RNA Concentration in Quant-iT™ RiboGreen® Assay
0	1,000	1,000	1 µg/mL
500	500	1,000	500 ng/mL
900	100	1,000	100 ng/mL
980	20	1,000	20 ng/mL
1,000	0	1,000	blank

Table 3. Protocol for preparing a low-range standard curve.

Volume (µL) of TE	Volume (µL) of 100 ng/mL RNA Stock	Volume (µL) of 2,000-fold Diluted Quant-iT™ RiboGreen® Reagent	Final RNA Concentration in Quant-iT™ RiboGreen® Assay
0	1,000	1,000	50 ng/mL
500	500	1,000	25 ng/mL
900	100	1,000	5 ng/mL
980	20	1,000	1 ng/mL
1,000	0	1,000	blank

1.5 Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus RNA concentration.

Sample Analysis

2.1 Dilute the experimental RNA solution in TE to a final volume of 1.0 mL in disposable cuvettes or test tubes. You may wish to prepare more than one dilution of the experimental sample.

High 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, then 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 **Eliminating DNA from Samples**.

2.2 Add 1.0 mL of the aqueous working solution of the Quant-iT™ RiboGreen® reagent (prepared above) to each sample. Incubate for 2 to 5 minutes at room temperature, protected from light.

2.3 Measure the fluorescence of the sample using instrument parameters that correspond to those used when generating your standard curve (see step 1.4). To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.

2.4 Subtract the fluorescence value of the reagent blank from that of each of the samples. Determine the RNA concentration of the sample from the standard curve generated in **RNA Standard Curves**.

2.5 The assay may be repeated using a different dilution of the sample to confirm the quantitation results.

Eliminating DNA from Samples

Quant-iT™ RiboGreen® reagent also binds to DNA. Fluorescence in samples that is due to Quant-iT™ 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.

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

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

3.3 Add ~5 units of RNase-free DNase I per µg of DNA estimated to be in the sample.

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

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

3.6 Perform the Quant-iT™ RiboGreen® assay as described above.

References

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

Product List **Current prices may be obtained from our Web site or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
R11490	Quant-iT™ RiboGreen® RNA Assay Kit	1 kit
R11491	Quant-iT™ RiboGreen® RNA reagent	1 mL
T11493	20X TE buffer *RNase free*	100 mL

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