Thermo SCIENTIFIC

PROTOCOL NanoDrop 3300

RiboGreen® Assay for RNA Quantitation

The RiboGreen® dye is a fluorescent nucleic acid stain for quantitating intact RNA. Used in conjunction with the micro-volume capability of the Thermo Scientific NanoDrop™ 3300 Fluorospectrometer, the RiboGreen® assay provides a highly sensitive means of RNA quantitation with minimal consumption of sample. The main disadvantage of general UV spectroscopy for RNA quantitation is the contribution of signal from degraded RNA and other contaminants, such as proteins and extraction buffers. RiboGreen® reagent circumvents such contributions from interfering substances by exhibiting an emission maximum at 530nm when bound specifically to intact RNA (unbound RiboGreen® reagent exhibits minimal fluorescence in solution). The ability of the NanoDrop 3300 to measure as little as 1 ul of sample, allows significantly scaled-down reaction volumes, thereby using only a fraction of sample commonly needed for conventional cuvette-based fluorometers. The NanoDrop 3300 has demonstrated a detection range for RNA of 25 ng/ml - 1000 ng/ml when using a high concentration of RiboGreen® dye (1:200 dilution), and 5 ng/ml - 50 ng/ml when using a 1:2000 dilution of RiboGreen® dye. (The following protocol is an adaptation of the Molecular Probes™ RNA Reagent Kit product information sheet.)

RiboGreen® Assay Supplies

Equipment:

- NanoDrop 3300 Fluorospectrometer
- 2uL pipettor (low retention nuclease free pipette tips)

Materials:

- Low lint laboratory wipes
- Nuclease free amber or foil covered 1.5 ml polypropylene tubes
- RNase inhibitor wipe or solution
- Polypropylene bottles, vials, and tubes

Reagents:

- RiboGreen® RNA reagent kit (Molecular Probes catalog # R11490) Includes 20X TE.
- DEPC treated water

RiboGreen® Assay Suggestions

- Aliquot dye concentrate into amber screw top tubes. Store at -20°C.
- Wipe down bench space, pipettes, and racks with RNase inhibitor before starting assay.
- Change gloves often to minimize nuclease contamination.

RiboGreen® Assay Protocol

1. Equilibrate the 20X TE buffer, RiboGreen® reagent (200X concentrate and DEPC treated dH_2O to room temperature. Protect from light.

2. Prepare 1X TE with DEPC treated water. The volume needed depends on the total number of samples to be measured and the volume of RiboGreen working solution.

3. Dilute the RiboGreen® Dye stock in two stages depending on RNA standard concentration range. (*Prepare fresh as the diluted dye is stable for only a few hours. Protect all stockes from light.*

   High RNA Quantitation range (25 ng/ml–1000 ng/ml)
   - Dilute the RiboGreen® stock (200X concentrate) by transferring 995 ul of 1X TE and 5 ul of the dye stock to a 1.5ml amber snap cap tube. Mix thoroughly.

   Low RNA quantitation range (5 ng/ml– 50 ng/ml)
   – Dilute the 1X dye in the previous step ten (10) fold further by transferring 900ul of 1X TE and 100ul of the 1X dye dilution to a 1.5ml amber snap cap tube,. Mix thoroughly.

4. Thaw the standard and unknown RNA samples. Once thawed, thoroughly mix each individual solution gently .

5. Prepare serially diluted RNA standards to 2X final concentrations in nuclease free vials or tubes (please refer to the example standard curve dilution series on page 2).

6. Aliquot one volume of diluted unknown RNA samples into appropriately labeled nuclease free amber tubes.

   Note: It is recommended that RNAs be diluted into 1X TE at an estimated 2x concentration which will fall in the middle of the standard curve.

7. Transfer an equal volume of the appropriate diluted RiboGreen® dye solution to each amber tube containing either the 2X standard RNA solution or unknown sample.

8. Prepare the Reference solution (negative control) by adding equal volumes of 1X TE and RiboGreen® working solution.

9. Mix each standard dilution and unknown sample thoroughly and allow to equilibrate at room temperature for 5 minutes. Proceed to Standard Curve Protocol (page 2).

Standard Curve Protocol

1. Clean both sampling pedestals with 2 uL of nuclease free deionized water.

2. Open upper arm and firmly blot the two pedestals with a dry lab wipe. Make sure there are no traces of lint on the pedestals before continuing.

3. Open the operating software. Click on the Nucleic Acid Quantitation button and select the RiboGreen method.

4. Add 2 uL of assay buffer (no dye, no sample) to the lower pedestal. Lower the arm and click F3 or the Blank button. When the measurement is complete, lift the arm and use a dry laboratory wipe to blot the buffer from both the bottom and upper measurement surfaces. Use a fresh aliquot of buffer to verify a proper baseline.

5. Under Measurement type, click on the Standards tab. Highlight the Reference standard.

6. Mix the reference solution (assay buffer and dye, no sample) briefly and transfer 2 uL of the solution onto the lower pedestal. Lower the arm and click F1 or the Measure button. A pop up window will ask for confirmation of the units. (Recommended ng/mL or pg/uL)

7. Measure up to 5 replicates of the reference solution using a fresh 2 uL aliquot for each measurement.

8. Select Standard 1 to enter a value. Enter values for up to 7 standards.

9. Mix the standard solution briefly and transfer 2 uL onto the lower pedestal. Lower the arm and click F1 or the Measure button. Measure up to 5 replicates of each standard using a fresh 2 uL aliquot for each measurement.

10. Once the standard curve is completed, select the Standard Curve Type (Interpolation, Linear, 2° polynomial, 3° polynomial) that best fits the standards data set.

11. Click on the Sample tab under Measurement Type, and enter the unknown samples' respective ID information. If a dilution of the unknown sample was made, enter the dilution factor in the box below the sample ID window.

12. Add 2 ul of the sample and use the F1 key or click the Measure button to initiate the measurement cycle. Use a fresh aliquot of sample for each measurement.

High RNA concentration range

Example of standard curve dilution series

Stock RNA (ng/ml)	2X RNA volume (ul)	1X TE buffer volume (ul)	2X RNA standard (ng/ml)	Final RNA (ng/ml)
2000	10*	0	2000	1000
2000	10**	10	1000	500
1000	10	10	500	250
500	10	10	250	125
250	5	20	50	25

Low RNA concentration range.

Example of standard curve dilution series

Stock RNA (ng/ml)	2X RNA volume (ul)	1X TE buffer volume (ul)	2X RNA standard (ng/ml)	Final RNA (ng/ml)
100	10*	0	100	50
100	20**	5	80	40
80	10	10	40	20
40	10	10	20	10
20	10	10	10	5

Example spectrum of RiboGreen RNA sample