EMORY UNIVERSITY

Emory Integrated Genomics Core

Emory Integrated Core Facilities

# EIGC.004_Quantitation with Fluorescence

## Standard Operating Procedure Staff Review Page:

**I have read and understand the procedure listed above.**

| Employee name | Date SOP review complete |
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# Standard Operating Procedure Approval Page:

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|---|---|---|
| Date Implemented: | 01 April 2006 | |
| Updated: | Ashima Amin<br>**Name** | 15 June 2020<br>**Date** |
| Supersedes: | Ashima Amin<br>**Name** | 11 April 2016<br>**Date** |

Annual Review and Approval

Michael Zwick, PhD
Laboratory Director

**Changes Made:**
061520: Review. SOP renumbered. Previously EIGC.005

***Changes to previous procedures:***041116: Review, LIMS steps updated, New instrument and protocol added, Tecan Infinite M200 Pro, with protocol for running, Old instrument and protocol removed (SoftMax Pro 5.2).

***Note: No laboratory personnel should use this piece of equipment without proper training by the laboratory supervisor or other designated person.***

***Test Principle:*** Fluorescence quantitation can be performed following DNA or RNA isolation. The most common use of fluorescence quantitation is for dsDNA, as fluorescence quantification is the most accurate method to quantify dsDNA in a DNA sample. DNA concentration is determined using Quant-it Broad Range and Quant-it High Sensitivity assays. The Quant-it PicoGreen assay of DNA is also available if the DNA will be used for Pyrosequencing. Fluorescence quantification of RNA is less common; however, the Quanti-it RiboGreen assay is a method of measuring RNA concentration by staining RNA with RiboGreen. Following fluorescence staining of the DNA or RNA, the sample is read using a plate reader to determine the concentration of the sample.

***Specimen Type:*** Isolated DNA or RNA

***Safety:***

All employees are required to follow universal safety protocols when working with potentially infectious biohazardous materials. Safety procedures are outlined in the Emory University Bloodborne Pathogen Exposure Control Plan (see Employee Safety Notebook).

As Quant-it reagents bind to nucleic acids, they should be treated as potential mutagens. DMSO solutions should be handled with caution as they are known to facilitate absorption into tissues.

***Quality Control:***

Standards are included with each run. Five out of seven standards must be within range for the data to pass. The $r^2$ value of the standard curve should not fall below 0.97. If the $r^2$ value is lower than 0.97 then samples and standards are re-plated and quantitated again. If the $r^2$ value fails a second time new standards or reagents should be used.

**Protocol:**

Carefully choose which quantitation is appropriate for the samples.

| | | |
|---|---|---|
| • Quant-iT PicoGreen. | 250 pg/mL-1000 ng/mL | dsDNA |
| • Quant-iT Broad Range | 2-1000 ng | dsDNA |
| • Quant-iT High Sensitivity | 0.2-100 ng | dsDNA |
| • Quant-iT RiboGreen High-Range | 20 ng/mL to 1 µg/mL | RNA |
| • Quant-iT RiboGreen Low-Range | 1 ng/mL to 50 ng/mL | RNA |

# Quant-iT PicoGreen Quantitation of DNA (for Pyrosequencing)

**Required Reagents:**

| | |
|---|---|
| 20X TE | Invitrogen – Cat# T11493 |
| Quant-iT PicoGreen Kit | Invitrogen – Cat# P7589 |
| Black 96-well plates | Greiner BioOne – Cat# 655076 |

**Protocol summary:**

1. Equilibrate assay components to room temperature.
2. Prepare a 2 µg/mL stock solution of DNA standard in 1xTE. Dilute λDNA (Component C) 50-fold.
3. Prepare serial dilutions for standard curve. **See table.**
4. Make the working solution by diluting Quant-iT™ PicoGreen reagent into 1xTE 200-fold (1 µL in 199ul).
5. Pipette 98 µL of 1xTE into each sample well.
6. Add 2 µL of each unknown DNA sample to separate wells and mix well
7. Add 100 µL of each of the λDNA standards to separate wells and mix well.
8. Load 100 µL of the working solution in each microplate well and mix well.
9. Incubate 2-5 minutes.
10. Measure fluorescence using microplate reader (excitation/emission maxima ~480/520 nm). See p9 for instructions.
11. Use a standard curve to determine DNA concentrations.

**Setting the standard curve:**

**5 Point Standard Curve with 2 µg/mL λDNA Stock**

| *Dilution* | *Method* | *[ ] with PicoGreen* | *Acceptable range* |
|---|---|---|---|
| 2.0 µg/mL | 20 µl stock + 980 µl 1X TE | 1.000 µg/mL | 0.998-1.003 µg/mL |
| 1.5 µg/mL | 15 µl stock + 985 µl 1X TE | 0.750 µg/mL | 0.745-0.755 µg/mL |
| 1.0 µg/mL | 10 µl stock + 990 µl 1X TE | 0.500 µg/mL | 0.495-0.505 µg/mL |
| 0.5 µg/mL | 5 µl stock + 995 µl 1X TE | 0.250 µg/mL | 0.200-0.300 µg/mL |
| 0.2 µg/mL | 100 µl 2 µg/mL + 900 µl 1X TE | 0.100 µg/mL | 0.098-0.102 µg/mL |

See **EIGC.004_Appendix_A** for detailed protocol process. Use 200ul volumes for 96-well plates. There are no deviations from published protocol. (MP 07581 Revised: 10-June-2008).

## Quantitation of dsDNA using Quant-IT Broad Range

**Required Reagents*:***

| | |
|---|---|
| Quant-iT Broad Range Kit | Invitrogen – Cat# Q-33130 |
| Black 96-well plates | Greiner BioOne – Cat# 655076 |

**Protocol summary:**

1. Equilibrate assay components to room temperature.
2. Make the working solution by diluting Quant-iT™ dsDNA BR reagent 1:200 in Quant-iT™ dsDNA BR buffer.
3. Load 200 µL of working solution in each microplate well that is being used.
4. Add 10 µL of each of the eight Quant-iT™ dsDNA BR standards to the appropriate microplate wells in Column 12, according to the template.
5. Add 2 µL of each unknown DNA sample to the appropriate microplate wells and mix well.
6. For controls
   a. Add 2 µL of 2-4 control DNAs with known concentration (select a range of DNA concentrations) to column 11.
   b. Do not add anything to the remaining wells in column 11. These will be NTCs (must have at least 1 NTC).
7. Measure fluorescence using microplate reader (excitation/emission maxima ~510/527 nm). See p9 for instructions.
8. Use a standard curve to determine DNA concentrations.

**Setting the standard curve:**

In order to obtain accurate DNA concentration reading, a standard curve must be run using DNA solutions of known concentrations. Each Broad Range kit comes with λDNA standards at a range of concentrations (0, 5, 10, 20, 40, 60, 80, and 100 ng/µL). A new standard curve must be performed for each run. Five out of six standards must fall on the standard curve to pass.

See **EIGC.004_Appendix_B** for detailed protocol process. Use 200 µL volumes for 96-well plates.
There are no deviations from published protocol. (MP 33130 Revised: 28-February-2007).

## Quantitation of dsDNA using Quant-iT High Sensitivity

**Required Reagents:**

| | |
|---|---|
| Quant-iT High Sensitivity Kit | Invitrogen – Cat# Q-33120 |
| Black 96-well plates | Greiner BioOne – Cat# 655076 |

**Protocol summary:**

1. Equilibrate assay components to room temperature.
2. Make the working solution by diluting Quant-iT™ dsDNA HS reagent 1:200 in Quant-iT™ dsDNA HS buffer.
3. Load 200 µL of working solution in each microplate well that is being used.
4. Add 10 µL of each of the eight Quant-iT™ dsDNA HS standards to the appropriate microplate wells, according to the template.
5. Add 2 µL of each unknown DNA sample to the appropriate microplate wells and mix well.
6. For controls
   a. Add 2 µL of 2-4 control DNAs with known concentration (select a range of DNA concentrations) to column 11.
   b. Do not add anything to the remaining wells in column 11. These will be NTCs (must have at least 1 NTC).
7. Measure fluorescence using microplate reader (excitation/emission maxima ~485/525 nm). See p9 for instructions.
8. Use a standard curve to determine DNA concentrations.

**Setting the standard curve:**

In order to obtain accurate DNA concentration reading, a standard curve must be run using DNA solutions of known concentrations. Each High Sensitivity kit comes with λDNA standards at a range of concentrations (0, 0.5, 1, 2, 4, 6, 8, and 10 ng/µL). A new standard curve must be performed for each run. Five out of six standards must fall on the standard curve to pass.

See **EIGC.004_Appendix_C** for detailed protocol process. Use 200 µL volumes for 96-well plates.
There are no deviations from published protocol. (MP 331020 Revised: 28-February-2007).

# Quantitation of RNA using Quant-iT RiboGreen

**Required Reagents:**

| | |
|---|---|
| 20X TE | Invitrogen – Cat# T11493 |
| RiboGreen Kit | Invitrogen – Cat# R11490 |
| Black 96-well plates | Greiner BioOne – Cat# 655076 |

Determine whether you wish to perform the **high-range assay** (20 ng/mL to 1 µg/mL RNA) or **low-range assay** (1 ng/mL to 50 ng/mL RNA), or both.

**Protocol summary:**

1. Equilibrate assay components to room temperature.
2. Prepare a 2 µg/mL solution of RNA standard in 1xTE. Dilute ribosomal RNA (Component C) 50-fold. Further dilute 20-fold to make 100 ng/mL for **low-range**.
3. Prepare serial dilutions for standard curve. See tables. Prepare fresh each time
4. Make the working solution by diluting Quant-iT™ RiboGreen reagent into 1xTE 200-fold for **high-range** and 2,000-fold for **low-range** assay.
5. Load 98 µL of 1xTE into each sample well.
6. Add 2 µL of each unknown RNA sample to separate wells and mix well
7. Add 100 µL of each of the ribosomal RNA standards to separate wells and mix well.
8. Load 100 µL of working solution in each microplate well.
9. Incubate 2-5 minutes.
10. Measure fluorescence using microplate reader (excitation/emission maxima ~480/520 nm). See p9 for instructions.
11. Use a standard curve to determine RNA concentrations.

**Setting the standard curve:**

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. Most single-stranded RNA molecules yield approximately equivalent signals. Choose either the **high-range (20 ng/mL to 1 µg/mL)** or **low-range (1 ng/mL to 50 ng/mL)** standard curve that is appropriate for the sample.

**High-Range Standard Curve**

| Volume (µL) of 1xTE | Volume (µL) of 2 ug/ml RNA Stock | Volume (µL) of 200-fold Diluted RiboGreen Reagent | Final RNA Concentration in Assay |
|---|---|---|---|
| 0 | 100 | 100 | 1 µg/mL |
| 50 | 50 | 100 | 500 ng/mL |
| 90 | 10 | 100 | 100 ng/mL |
| 98 | 2 | 100 | 20 ng/mL |
| 100 | 0 | 100 | blank |

**Low-Range Standard Curve**

| Volume (µL) of TE | Volume (µL) of 100 ug/ml RNA Stock | Volume (µL) of 2000-fold Diluted RiboGreen Reagent | Final RNA Concentration in Assay |
|---|---|---|---|
| 0 | 100 | 100 | 50 ng/mL |
| 50 | 50 | 100 | 25 ng/mL |
| 90 | 10 | 100 | 5 ng/mL |
| 98 | 2 | 100 | 1 ng/mL |
| 100 | 0 | 100 | blank |

See **EIGC.004_Appendix_D** for protocol process. Use 200 µL volumes for 96-well plates. There are no deviations from published protocol. (MP 11490 Revised: 10-June-2008)

## Reading the Nucleic Acid concentration

1. Open “Magellan v 7.1” Software on the Tecan Infinite M200 Pro.
2. Create / Edit a Method
3. Open Template for Assay you are running. C:\users\public\Documents\Tecan\Magellan\mth – Templates DO NOT EDIT-DO NOT SAVE)
4. Choose Measurement Parameters
   a. Plate Layout – Fill wells with samples (SM-sample, BL-blanks, ST-standards).
   b. Make sure dilutions are correct and standards have the correct concentrations.
   c. Standards Curve should be set to linear regression.
5. Save template with Project ID, order number, and date.
6. *Templates from two plates can be added at the same time, for two plates that would be run one after another.
7. Finish and Run.
8. Place the incubated black plate in the plate reader drawer then Read.
9. When all samples have been read, add Sample IDs into the excel file. Add the quantifications to the QC Report or Project Summary for the order. Save the files on PPMS. These concentrations can also be added to the LIMS ID’s by ‘modifying their aliquots’ and entering the values into the Concentration field.

***Benefits and Limitations:***

This procedure requires at least 3 μL of extracted DNA/RNA solution.

It is recommended that all fluorescent solutions be used within a few hours of its preparation, and that it be covered with foil as the fluorescent reagent is susceptible to photodegradation.

***Troubleshooting:***

See user’s manual