STANDARD OPERATING PROCEDURE (SOP)

SOP Title:	**CAGE QUANT-IT OLIGREEN SSDNA ASSAY (WEB VERSION)**
SOP Number:	SOP002-02
Effective Date:	25 APR 2020
Current Review Date:	25 APR 2022
Replaces SOP Number:	SOP002-01
Group:	GIH

I have read this document and approve its contents.

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A. PURPOSE AND APPLICATION

This SOP covers how to quantify single strand (c)DNA using an ultra-sensitive fluorescent nucleic acid stain at day 4 of the commercial CAGE protocol (Day 2 of the in-house CAGE protocol). This is part of a quality check after cap-trapping of 5' end of RNA transcripts, prior to linker ligation. Expected yield of cDNA is 15 – 30 ng/sample although the amount of ss cDNA may be different depending on the source of RNA samples used.

B. BRIEF SUMMARY OF METHOD

The Quant-iT Oligreen ssDNA Reagent and kit (Catalog no. 011492) is used to quantify ss cDNA according to manufacturer's instructions. The kit is supplied with 1ml of Quant-iT Oligreen ss reagent solution, 20X TE and oligonucleotide standard. Each standard and diluted ssDNA are mixed with aqueous working solution of Quant-iT Oligreen reagent and the fluorescence is measured using a spectrofluorometer and standard fluorescein wavelengths (excitation ~480 nm, emission ~ 520 nm).

C. DEFINITIONS AND ABBREVIATIONS

CAGE – Cap Analysis Gene Expression, a method of generating sequence data for 5' end of RNA transcripts.

D. OCCUPATIONAL HEALTH AND SAFETY

No data are currently available addressing the mutagenicity or toxicity of Quant-iT™ OliGreen® reagent.

This reagent binds to nucleic acids, and therefore should be treated accordingly 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. It is strongly recommended to use double gloves when handling the DMSO stock solution.

E. CAUTIONS

The Oligreen reagent is light sensitive. Protect the reagent from light as much as possible during this procedure. Ideally perform the experiment in the dark and do not over-incubate with Quant-iT Oligreen reagent before measuring fluorescence. Prepare the Oligreen reagent in plastic tubes, as the reagent may adsorb to glass surfaces.

F. PERSONNEL QUALIFICATIONS, TRAINING AND RESPONSIBILITIES

Training Requirements:

[X] Read and Understand Document [X] Training Required

GIH utilises the Fluroskan spectrofluorometer belonging to the PCTG group to perform this procedure. If you wish to use this instrument, you must seek permission and training before beginning the experiment (please coordinate this with PCTG group manager, Anjali Henders or lab manager Leanne Wallace).

G. EQUIPMENT AND MATERIALS

Equipment

a. Pipettes – P10, P20, P200, P1000. For many samples, multichannels may be useful
b. Spectrofluorometer
c. Benchtop centrifuge for 1.5 ml tubes

Materials

a. Quant-iT Oligreen ssDNA Reagent and kit (Life Technologies, O11492)
b. Pipette tips – P10, P20, P200, P1000, plus multichannel tips as needed
c. 1.5 ml LoBind tubes
d. 15 ml tubes
e. 96 well optiplate (Perkin Elmer, 6005270)
f. UltraPure water (ThermoFisher; 10977015)
g. Aluminium Foil

H. PROCEDURE

- Prepare assay buffer:

TE buffer is used for diluting the Quant-iT Oligreen reagent and for diluting oligonucleotide and ss DNA samples. Because the Quant-iT Oligreen reagent is an extremely sensitive detection reagent for ss DNA, it is imperative that the TE solution used is free of contaminating nucleic acids. The 20X TE buffer that is included in the assay kit is nuclease-free and nucleic acid-free. Prepare the 1X TE working solution by diluting the concentrated buffer 20-fold with sterile, distilled, DNase-free water.

- Prepare Oligreen reagent:

Allow the reagent to warm to room temperature before opening the vial. Immediately before the experiment, prepare an aqueous working solution of the Quant-iT Oligreen reagent by making a 200-fold dilution of the concentrated solution in TE buffer. You will require 100µl of diluted reagent per well. For the standard curve consisting of 5 points in quadruplicate, 2mL of diluted reagent is needed. Each sample performed in triplicate will require an additional 320µl of diluted reagent. It is recommended to include at least 5% overfill to ensure adequate volume to complete the assay.

For example, for 8 samples, add 25 µl Oligreen reagent to 4.975 µl 1X TE buffer. Prepare this solution in a 15 ml plastic tube and protect the working solution from light by covering it with foil. For best results, use this solution within a few hours of preparation.

- Prepare oligonucleotide standards:

Dilute oligonucleotide standard, provided at 100 µg/ml in the Oligreen Assay Kit, 50-fold in 1X TE buffer to make the 2 µg/ml working solution (i.e. 2 µl of the standard in 98 µl of TE buffer).

Prepare a 20-fold dilution of the 2 µg/mL oligonucleotide solution to yield a 100 ng/mL oligonucleotide stock solution (i.e. 30 µl of the 2 µg/ml working solution in 570 µl of TE buffer). For the low-range standard curve (from 100 pg/ml to 50 ng/ml), dilute the 100 ng/mL oligonucleotide stock solution into 1.5 ml tubes as shown below.

Volume of TE buffer	Volume of 100 ng/mL oligomer stock	Volume of diluted Quant-iT Oligreen reagent	Final oligomer concentration in Quant-iT Oligreen Assay
0 µL	500 µL	500 µL	50 ng/mL
450 µL	50 µL	500 µL	5 ng/mL
495 µL	5 µL	500 µL	500 pg/mL
499 µL	1 µL	500 µL	100 pg/mL
500 µL	0 µL	500 µL	blank

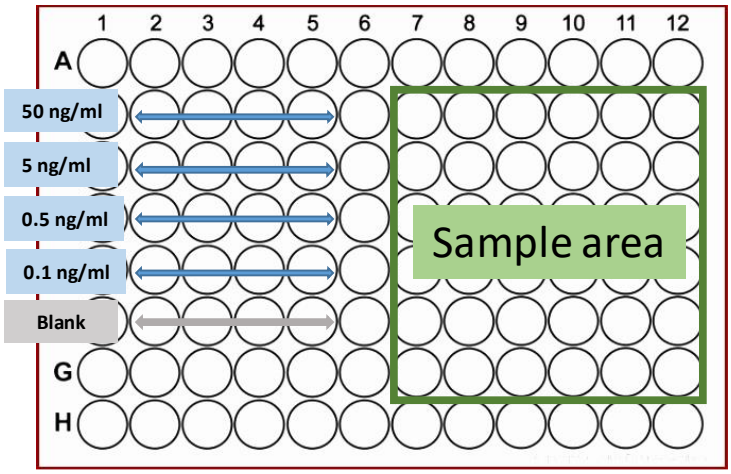
- Prepare sample dilutions:

Prepare 3 replicates of ss DNA sample at 1 in 100 dilution with TE buffer (i.e. 3.2 µl of ss DNA in 316.8 µl of TE). Add equal volume of the aqueous working solution of the Quant-iT Oligreen reagent (i.e. 320µl) to each sample.

- Set up assay:

Dispense 200 µL of each prepared oligonucleotide standard into appropriate wells (4 replicates for each standard).

Dispense 200 µL of each prepared sample into appropriate wells (3 replicates for each sample). See below for an example plate setup.



Transfer the plate into Fluoroskan Micoplate Fluorometer.

Shake the plate for 1 min at 600 rpm (shaking type: Continuous, Shaking speed and force: Medium).

Incubate (pause) for 3 min at room temperature.

Measure the sample fluorescence using standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nM).

Use the instrument software or other preferred method to calculate the sample concentrations according to the standard curve.

I. WORKED EXAMPLE

RNA samples used for CAGE pilot project (ESC1041, 1067, 1271, 1369) were reverse transcribed to ss cDNA and quantified for QC testing at day 4 of CAGE analysis. The data from the Quant-iT Oligreen ss DNA quantification assay are available as a positive control for further CAGE experiment and are as follows:

	ng/ml	X100 for dilution factor	pg/ul	Total ssDNA (pg) in 50 ul	In ng
ESC1041	3.34	334	334	16700	16.7
ESC1067	3.42	342	342	17100	17.1
ESC1271	3.72	372	372	18600	18.6
ESC1369	3.40	340	340	17000	17

Expected yield of ss cDNA post cap-trapping is 15 – 30 when 5 µg of RNA/sample is used.

J. SOP VALIDATION DETAILS

This SOP has been developed according to manufacturer's instructions. The ssDNA samples that were quantified with this method were continued to final library prep according to the in-house and the commercial CAGE protocols ~~(SOP001-01)~~. The generated libraries were sequenced and analysed by Dr. Quan Nguyen, an expert in CAGE analysis, and were judged to be of good quality.

K. WASTE MANAGEMENT AND DISPOSAL

Microcentrifuge tubes with residual Oligreen reagent and optiplate containing the oligreen components are to be disposed of into clinical waste bins according to IMB waste management protocol. Sharps are to be disposed of into puncture-resistant clinical sharps bins. There are no special waste disposal requirements associated with this SOP.

L. DATA RECORDS MANAGEMENT

We recommend storing a copy of the exported excel spreadsheet, and employing a sample tracking system that can easily link this document back to each individual experiment.

M. REFERENCE DOCUMENTS

The Quant-iT Oligreen ssDNA kit user guide developed by the manufacturer can be found here: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FQuant_iT_OliGreen_ssDNA_Reagent_UG.pdf&title=VXNlciBHdWlkZTogUXVhbnQtaVQgT2xpR3JlZW4gc3NETkEgUmVhZ2VudCBhbmQgS2l0

Risk assessments associated with this SOP are available in the IMB Risk Management Database in WebDB:

- Risk Assessment ID #2591 “Cap-analysis gene expression (CAGE) with CAGE Library preparation kit and in-house protocol”. A pdf version of this risk assessment is available on the CAGE development page of the GIH website.

Other SOPs referenced in this SOP are available on the CAGE development page of the GIH website:

- SOP006-02 in-house CAGE

N. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA) SECTION

Variability of replicates of standards and samples and linearity of the standard curve should be assessed to determine validity of data.