STANDARD OPERATING PROCEDURE (SOP)

SOP Title:	**CAGE rRNA CONTAMINATION qPCR (WEB VERSION)**
SOP Number:	SOP003-02
Effective Date:	25 APR 2020
Current Review Date:	25 APR 2022
Replaces SOP Number:	SOP003-01
Group:	GIH

I have read this document and approve its contents.

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SOP003-02	Sohye Yoon/Stacey Andersen	25 APR 2020

A. PURPOSE AND APPLICATION

This SOP covers determination of the extent of rRNA contamination in ss cDNA samples by quantitative PCR, after cap-trapping of 5' end transcripts during both in-house CAGE and commercial CAGE. This procedure is critical to determine how effectively the cap-trapping step has been performed.

B. BRIEF SUMMARY OF METHOD

Diluted ss cDNA samples after cap-trapping are used as a template for quantitative PCR analysis. A diluted cDNA sample is amplified using both rRNA and ACTB primers and the Ct (Cq) difference between the two primer sets are obtained to determine rRNA contamination in the samples. The expected delta Ct is 2 – 4 cycles (ACTB-rRNA).

C. DEFINITIONS AND ABBREVIATIONS

ss cDNA : single strand cDNA
CAGE: Cap Analysis Gene Expression
rRNA: ribosomal RNA
ACTB: Actin Beta (β)

D. OCCUPATIONAL HEALTH AND SAFETY

Standard PC2 laboratory guidelines and procedures are to be adhered to while performing this protocol.

E. CAUTIONS

SYBR master mix is sensitive to light. Protect the reagent from light as much as possible during this procedure.

F. PERSONNEL QUALIFICATIONS, TRAINING AND RESPONSIBILITIES

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

G. EQUIPMENT AND MATERIALS

Equipment

a. Pipettes – P10, P20, P200, P1000. For many samples, multichannels may be useful.
b. qPCR machine for 96 well plate (384 well plate if >8 samples)
c. Benchtop centrifuge for 1.5 ml tubes
d. Vortex
e. Plate centrifuge

Materials

a. SYBR master mix
b. Nuclease-free 96 well PCR plates
c. Optically-clear plate seals
d. UltraPure water (ThermoFisher; 10977015)
e. Pipette tips – P10, P20, P200, P1000, plus multichannel tips as needed
f. 1.5mL LoBind tubes
g. PCR plate cooler
h. Validated qPCR primers for ACTB and rRNA (see below for primers for use with human samples)
   - Hs_ACTB_qPCR_F – GGCATGGGTCAGAAGGATT
   - Hs_ACTB_qPCR_R – AGGTGTGGTGCCAGATTTTC
   - Hs_rRNA-qPCR_F – CTGGTTGATCCTGCCAGTAG
   - Hs_rRNA-qPCR_R - TCTAGAGTCACCAAAGCCGC

H. PROCEDURE

- Sample preparation

  For Commercial CAGE – At day 4, make 1 in 30 dilution of single strand DNA samples.
  For in-house CAGE – At day 2, make 1 in 30 dilution of single strand DNA samples.

- Procedure

1. Prepare the required volume of master mix **for each primer pair** using the reaction setup recommended below. Make enough master mix for each sample in triplicate, no template control (NTC) in triplicate, plus overfill.

Reagent	Volume (µl)
2X SYBR Green master mix	5
Forward + reverse primer mix (10 µM each)	1
Total volume	6

2. Pipette ix and briefly centrifuge the reagent master mix.

3. Dispense 6 µl of the master mix into wells of 96 or 384 well plate, depending on number of samples (3 replicates/sample + 3 replicates/NTC).

4. Add 4 µl of UltraPure water to all NTC wells.

5. Dispense 4 µl of each diluted ss cDNA into the appropriate well (3 replicates/sample/primer pair).

6. Seal the PCR plate, vortex to mix, spin down briefly and transfer to the qPCR instrument.

7. Perform qPCR with the following cycling protocol.

Step	Temp. (°C)	Duration	Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Anneal/Extension	60	30 sec	
Melting curve : 65 – 95 °C (optional)			

I. WORKED EXAMPLE

All post cap-trapping cDNA samples generated for the CAGE project have been assessed with this procedure. This quantification analysis data is available as a positive control for QC testing for further implementation of this protocol.

Below is an example from one of the CAGE pilot experiments:

- Check rRNA contamination by qPCR:

	Mean Ct of rRNA	Mean Ct of ACTB	Delta Ct (ACTB-rRNA)
ESC1041	14.31	16.47	2.16
ESC1067	14.66	16.51	1.85
ESC1271	14.15	16.69	2.54
ESC1369	14.53	16.57	2.04

Note: ACTB - rRNA ΔCT at ssDNA QC check 1 is expected to be <4

J. SOP VALIDATION DETAILS

This SOP has been developed according to manufacturer's instructions. The generated libraries were sequenced and analysed by Dr. Quan Nguyen, an expert in CAGE analysis, and have been judged to be of good quality (total proportion of rRNA was determined to be less than 1 % of total mapped reads).

K. WASTE MANAGEMENT AND DISPOSAL

Microcentrifuge tubes with residual master mix and qPCR plate containing PCR 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 both the .eds instrument file and the exported results file containing the ΔCt calculations, and employing a sample tracking system that can easily link these documents back to each individual experiment.

M. REFERENCE DOCUMENTS

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

Review the amplification curves and the Ct scores for replicate data points (ssDNA and controls), and exclude obvious outliers. Replicate data points should differ by ≤0.2 cycles.