STANDARD OPERATING PROCEDURE (SOP)

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A. PURPOSE AND APPLICATION

This SOP describes quantification of CAGE libraries using KAPA library quantification system to determine concentration of sequencing-capable fragments in the final libraries. The KAPA library quantification kit for Illumina platforms provides all the reagents needed for absolute, qPCR-based quantification of Illumina libraries flanked by the P5 and P7 flow cell oligo sequences. This SOP covers quantification of both in-house and commercial CAGE libraries. Different dilution factors are required for the different library types as the in-house CAGE protocol involves library amplification while the commercial CAGE protocol is a PCR-free procedure.

B. BRIEF SUMMARY OF METHOD

Diluted libraries are quantified by qPCR using 2X KAPA SYBR FAST qPCR master mix with primer premix. The concentration of the library will be determined by a DNA standard curve that is generated using amplification data of 6 DNA standards.

C. DEFINITIONS AND ABBREVIATIONS

CAGE – Cap Analysis Gene Expression

D. OCCUPATIONAL HEALTH AND SAFETY

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

E. CAUTIONS

Any library dilution that amplifies before DNA Standard 1 should not be used in library concentration calculations. Any library dilution that amplifies within 5 Ct of a no-template control should not be used in library concentration calculations. In these cases, quantification should be repeated with adjusted dilutions. Choice of library dilutions should be based on estimations from previous experience with libraries of the same type, or prepared using similar workflows, and/or on concentration information obtained with other methods during library construction and quality control (e.g., with NanoDrop™, Qubit® or Bioanalyzer).

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. Benchtop centrifuge for 1.5mL tubes

c. Centrifuge for multiwell plates
d. qPCR machine for 96 well plate (384 well plate if >8 samples)
e. PCR plate cooler

Materials

a. Pipette tips – P10, P20, P200, P1000, plus multichannel tips as needed
b. 1.5mL LoBind tubes
c. Nuclease-free 96 well PCR plates
d. Nuclease-free 384 well PCR plates (for >8 samples)
e. Optically-clear plate seals
f. UltraPure water (ThermoFisher; 10977015)
g. KAPA Library Quantification Kit for Illumina (KAPA Biosystems; KK4835)

H. PROCEDURE

- If performing procedure at the IMB: book appropriate PCR instrument through https://bookings.imb.uq.edu.au/Web/schedule.php (Real Time PCR section).

- Sample preparation:

For Commercial CAGE – At day 8, make 1 in 1000, 5000, 10000, 20000 dilutions of double strand cDNA library.

For in-house CAGE – At day 5, make 1 in 10000, 50000, 100000, 200000 dilutions of amplified double strand cDNA library.

Note: Once user is familiar with the particular library prep protocol, 2 dilutions is sufficient for quantification. Ideally, choose dilutions which fall within the range of the standards 1-4.

- Procedure

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

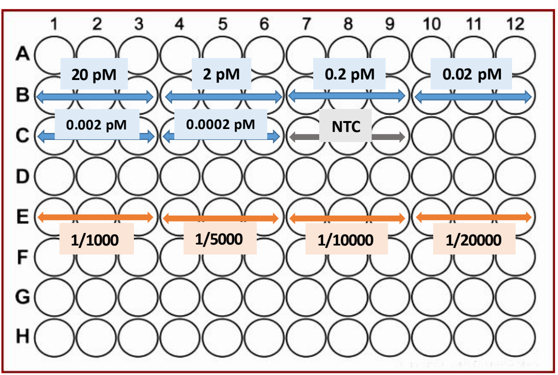
Reagent Volume	(µl)
2X SYBR Green master mix	5
Primer premix	1
H_2O	2
Total volume	8

2. Pipette mix and briefly centrifuge the reagent master mix.
3. Dispense 8 µl of the master mix into wells of 96 or 384 well plate, depending on number of samples (3 replicates/standard + 3 replicates/sample + 3 NTC).
4. Add 2 µl of UltraPure water to all NTC wells.
5. Dispense 2 µl of each DNA standard into the appropriate well, working from the most dilute (Standard 6) to the most concentrated (Standard 1).

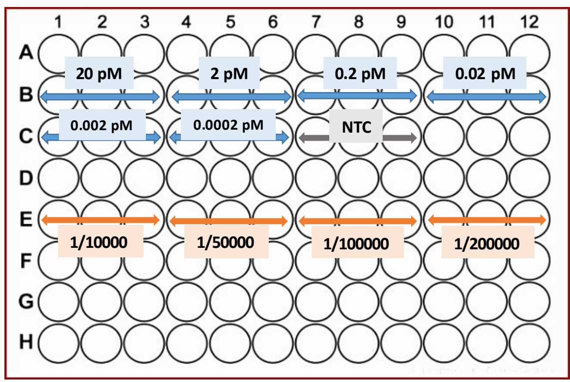
DNA Standard 1	20 pM
DNA Standard 2	2 pM
DNA standard 3	0.2 pM
DNA standard 4	0.02 pM
DNA standard 5	0.002 pM
DNA standard 6	0.0002 pM

6. Dispense 2 µl of each library dilution into the appropriate well. See below for example plate setups.

a) For commercial CAGE:



b) For in-house CAGE:



7. Seal the PCR plate, vortex to mix, spin down briefly and transfer to the qPCR instrument.
8. Perform qPCR with the following cycling protocol.

Step	Temp. (°C)	Duration	Cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	40
Anneal/Extension	60	2 min	
Melt: 65°C – 95 °C (optional)			

- Analysis

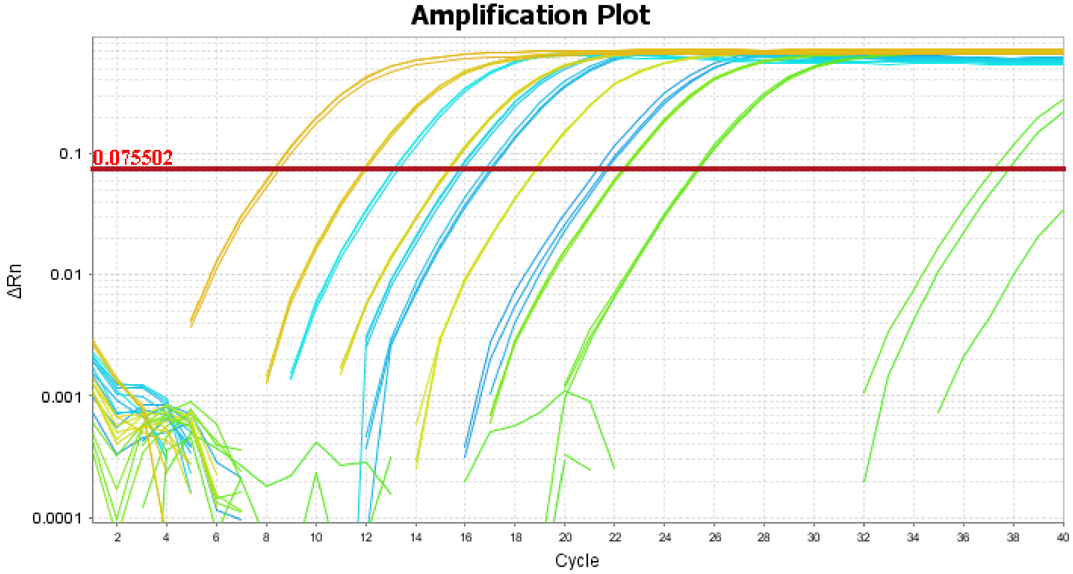
Ct data obtained from the instrument .eds file using QuantStudio software can be copied into the KAPA Library Quant Data Analysis Template available on the CAGE development page of the GIH website.

With addition of average library fragment length determined by BioAnalyser, also performed as part of final library QC for both commercial and in-house CAGE protocols, this spreadsheet will calculate molar concentrations for libraries. Instructions for use are included on Sheet 1 of the document.

I. WORKED EXAMPLE

All libraries generated for the CAGE project have been quantified with this procedure. This quantification analysis data is available as a positive control for QC testing for further KAPA library quantification.

As an example, a qPCR amplification plot with 6 standards and a single pooled commercial CAGE library is shown below. Pooled concentration (for 4-plex library) is 200pM in 10uL final volume.



J. SOP VALIDATION DETAILS

The SOP has been developed according to manufacturer’s instructions. Libraries quantified using this method have been loaded onto the Ilumina NextSeq instrument at recommended concentrations and produced expected cluster densities. The quantified libraries using this SOP 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 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 completed KAPA Library Quantification Analysis Template, 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

1. Review Ct values for the six DNA standards, to evaluate the reliability of the data:

- If the Ct value for any replicate varies by more than +/- 0.25 of a cycle from the other value(s) (as calculated in column I), that replicate should be regarded as an outlier. The difference of 0.25 cycles is arbitrary and instrument-dependent. When deciding on what to classify as an outlier, keep in mind that 1 cycle represents a 2-fold difference in concentration.

- The average Ct value for each DNA Standard should be ~3.3 cycles later than the DNA Standard that is 10-fold more concentrated (between 3.2 and 3.45 is very good, whereas 3.1 - 3.6 is acceptable.

- The Delta Ct between no template control (NTC) reactions and DNA Standard 6 should preferably be ≥3 cycles.

2. When standard curve is generated In KAPA-Library-Quant_Data-analysis template, the efficiency should be between 90 and 110% and R^2 should be between 0.99 and 1.00.