Document Number:	**SASoM/METHOD/075.v4**
Title:	**GeCKO lentiCRISPRv2 library DNA amplification in Lucigen bacteria**
Version:	**v4**
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SOP History		
Number	Date	Reason for Change
v1	27/10/2014	Original
v2	10/10/2016	Update
v3	10/10/2018	Update
V4	11/11/2020	Update

1.0 Purpose –
This SOP describes the current procedure for GeCKO lentiCRISPRv2 library DNA amplification in Lucigen bacteria for use in Laboratory 248K at the St Andrews School of Medicine (SASoM).

2.0 Scope –
This SOP applies to the staff in the SASoM involved in GeCKO lentiCRISPRv2 library DNA amplification in Lucigen bacteria.

3.0 Responsibilities –
All staff involved in cell culture are responsible for ensuring that the methods are followed in accordance with this SOP.

All staff must have read and signed the relevant Risk Assessment(s) documents before performing this procedure.

4.0 Procedure –

This procedure is for amplifying DNA from the GeCKO sgRNA library (Addgene). The library is delivered as two half-libraries (A and B). When used together, the A and B libraries contain 6 sgRNAs per gene (3 sgRNAs in each library). Both A and B libraries contain 1000 control sgRNAs designed not to target in the genome. The A library also targets miRNAs (4 sgRNAs per miRNA). The library must be amplified by bacterial electroporation and by growth of colonies on solid medium, not liquid culture in order to maintain library coverage.

GeCKO pooled electroporation, plating, determination of transformation efficiency and maxi prep. Follow these instructions for each library (A and B). Amplify and prep half-libraries A and B **separately** as follows:

For each half-library, you will need the following:

(i) twenty-three (23) x LB Agar-Carbenicillin petri dishes,
(ii) Sterile culture tubes (n=5)
(iii) Endura electropetent cells – 4 aliquots (from 2 tubes of 50µl)
(iv) 1.5mL sterile microcentrifuge tubes (4)
(v) Cuvette for electroporation (4).

Protocol:

1. Preparation of LBAgar-Carbenicillin.(day before)>23 x LB Agar-Carbenicillin petri dishes (10cm) – get about 8 plates from 300mL LBAgar. Don't pour too thin. Make a few extra than needed. LBAgar is made from adding water to powder as directed by manufacturer (Sigma) and autoclaving on the same day. To make plates, microwave bottle of LBAgar at low power to melt, then leave to cool to "hand-hot" before adding appropriate antibiotic and pouring plates.

2. Dilute the GeCKO library to 50 ng/µL in water or TE (if not already diluted).

3. Electroporate the library

   a. Add 2 µL of 50 ng/µL GeCKO library to 25 µL of Lucigen Endura electrocompetent cells (Cambridge Bioscience: #60242) with an efficiency of ≥10^9 cfu/ per µg DNA. (>1500 colonies on 1:40,000 dilution plate – see below).
   b. Electroporate using the manufacturer's suggested parameters/protocol (see below).

Transformation is carried out in a 1mm gap cuvette (VWR: #732-1135) using Bio-Rad Micro Pulser #165-2100 (Malcolm White Lab, BSRC Annex 3rd Floor).

Specifications: Program EC1, 1.8kV, 5.4ms pulse duration.

Optional transformation control reactions include electroporation with 1 µL (10 pg) of supercoiled pUC19 DNA.

1. Place bacterial cells, cuvettes and eppendorfs on ice
2. Let bacteria cells thaw on wet ice until they thaw completely (10-20 minutes). When cells are thawed, mix them by tapping gently. Aliquot 25 µL of cells to the chilled microcentrifuge tubes on ice.
3. Thaw recovery media and leave at RT, label sterile culture tubes and leave at RT.
4. Add 2µL GeCKO library DNA to bacteria cells and mix gently with the yellow tip
5. Carefully pipet 25 µL of the cell/DNA mix into a chilled electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward with your wrist to deposit the cells across the bottom of the well (the inside floor is about half way up the cuvette).
6. Set up electroporator: choose Program EC1.
7. Place the cuvette in the electroporator without cap (notch facing electrode unit)
8. Press Pulse button (PLS appears on screen + beep sound)
9. Press measurements button for verification of voltage delivered (1.8kV)
10. Press measurements button again for verification of time delivered (5.4ms)

c. Within 10s after Pulse, recover with 975 µL recovery media (i.e. media provided with cells) and transfer to a loosely capped tube containing an additional 1 mL of recovery media.

d. Repeat for a total of 4 electroporations and rotate at 250 rpm for 1 hr at 37°C Pre-warm 23 LBAgar + carbenicillin (100 µg/mL) 10cm dishes in microbiology incubator at 37°C.

4. Plate a dilution to calculate transformation efficiency. *Note the library plasmids have **ampicillin resistance** – prepare all plates accordingly.*

a. Pool all 8 mL of electroporated bacterial cells together. Mix well.

b. Remove 10 µL and add to 1 mL of recovery media, mix well, and plate 20µL (in around 100uL LBroth) onto a pre-warmed 10cm petri dish (carbenicillin). This is a 40,000-fold dilution of the full transformation and will enable you to estimate transformation efficiency to ensure that full library representation is preserved. Repeat for 2 more plates, so count three plates in total and calculate the average number.

5. Plate the transformations
   a. Spread 400 µL of transformation mix per petri onto 20 pre-warmed petri dishes (carbenicillin).

6. Grow all plates inverted for **14 hr** at **32°C**. Growth at this lower temperature reduces recombination between the lentiviral long-terminal repeats. (Growth at 37°C is also acceptable if 32°C is not possible.)

7. Calculate transformation efficiency

   a. Count the number of colonies on the dilution plate.

   b. Multiple this number of colonies by 40,000 for the total number of colonies on all plates.

   c. Proceed if the total number of colonies is at least 6×10^7. This efficiency is equivalent to 50 colonies per construct in the GeCKO library.

8. Harvest colonies

   a. Pipette 500 µL of LB Broth onto 10 cm petri dish

   b. Scrape the colonies off with a cell spreader/scraper.

   c. Pipette off the liquid plus scraped colonies into a tube and repeat the procedure a second time on the same plate with additional 500 µL of LB Broth. Note: Weigh this tube prior to adding any liquid to it.

9. Weigh the bacterial pellet to determine the proper number of maxiprep columns to use

   a. Spin down all liquid to pellet the bacteria and then discard the supernatant.

   b. Weigh the bacterial pellet and subtract the weight of the tube

10. Maxiprep for downstream virus production and future amplification

   a. Using a maxi scale plasmid prep, each column can handle approximately

0.45 g of bacterial pellet.

b. Perform a sufficient number of maxi preps so as to not overload a column. Around 8 maxipreps are expected.

11. Proceed to transient transfection of 293T(SD) cells with maxi-prepped GeCKO lentiCRISPRv2 library and appropriate packaging plasmids.

If producing virus from the full library, transfect cells with both A and B half-library plasmids separately and then pool viral particles for recipient cell infection.

5.0 Personal protection -
A Howie laboratory coat and lab gloves must be worn at all times.

6.0 Spillages -
Always clean up any spills immediately after use, only you know what you have spilt and are aware of its hazard.

Spillages should be mopped up with paper towel, disinfected with 70% ethanol and finally washed with water.

7.0 Training -
All staff should be trained in microbiological technique.

8.0 Related documents –

8.1 Risk assessments –
RA-BIOL-001-Handling of bacteria

RA/BIOL/004-Tissue culture

RA/GM/002 Viral-mediated gene delivery into mammalian cells

8.2 SOPs –
SASoM-METHOD-075-GeCKO lentiCRISPRv2 library DNA amplification in Lucigen bacteria.

SASoM-METHOD-077-GeCKO genomic DNA purification Qiagen Midi kit.

SASoM-METHOD-078-Transduction of GECKO Library in MiaPaCa2 cells.

SASoM-METHOD-079-DNA quantification using QubiT fluorometer 2.0 for GeCKO library.

9.0 Approval and sign off –

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