Document Number: **SASoM/METHOD/080.v5**

Title: **PCR for GeCKOv2 library preparation for next-generation sequencing**

Version: **v5**

Author: **Paul Reynolds**

Effective from:	05/08/2021
Valid to:	05/08/2023

SOP History		
Number	Date	Reason for Change
v1	02/04/2015	Original
v2	05/08/2015	Minor Amendments
v3	05/08/2017	Update
V4	05/08/2019	Update
V5	05/08/2021	Update

1.0 Purpose –

This SOP describes the current procedure for performing PCR reactions on the GeCKOv2 library for Next generation sequencing in Laboratory 248 at the St Andrews School of Medicine (SASoM).

2.0 Scope –

This SOP applies to the staff in the SASoM involved in genome-wide loss of function screening using GeCKOv2 library.

3.0 Responsibilities –

All staff 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 documents before performing this procedure.

4.0 Procedure –

This procedure is a **2-Step PCR** protocol using Herculase II Fusion DNA Polymerase.

For the first PCR, the amount of input genomic DNA (gDNA) for each sample was calculated in order to achieve 300X coverage over the GeCKO library (assuming 6.6µg of gDNA for 10^6 cells). For each sample, perform PCR reactions (100µl) containing 10µg genomic DNA in each reaction.

DNA input needed= 260µg for each sample

For **each sample,**

PCR 1

26 reactions of **100µl** (= 2.6ml) containing **10µg DNA** each are needed per biological sample. Determine the required volume of (i) DNA template to get 300 µg (30 reactions x 10 µg) and (ii) dH_2O.

Prepare a Master mix for 30 reactions *without DNA template and Herculase* for each sample. Add the components (in the prescribed order – see table below) in a ~5ml tube while mixing gently.

Quantity for 1 reaction (100µl) Quantity for 30 reactions (total 3ml)

Components	Targets < 1 kb
dH2O	Xµl to final vol
5X Herculase buffer	20µl
dNTPs Mix (25 mM each)	1µl
DNA template	*Xµl (10µg)*
Primer 1	2.5µl
Primer 2	2.5µl
Herculase DNA Polymerase	*1µl*
DMSO	3µl
Total reaction volume	100µl

30 reactions
Xµl to final volume
600µl
30µl
Xµl (300µg)
75µl
75µl
30µl
90µl
3ml

Put 100 µl – (DNA template volume and Herculase volume) of master mix in 27 sterile 0.2 ml thin walled PCR tubes per sample.

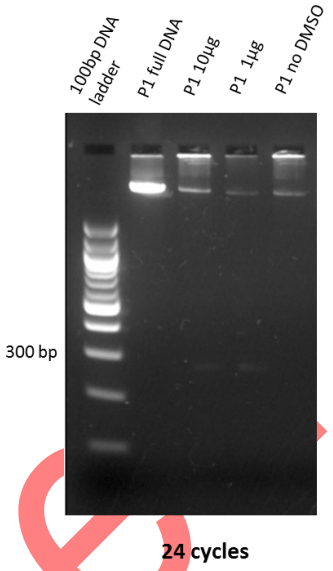
Add DNA template in 26 of the PCR tubes and an equal volume of dH_2O (= control) in 1 tube.

Add 1 µl Herculase in each tube.

PCR conditions on G-Storm thermal cycler (Lab 249)

PCR Conditions			
Initial denaturation	95˚C	2 min	1 cycle
Denaturation Annealing Elongation	95˚C 52˚C 72˚C	15 s 20s 30s	24 cycles
Final extension	72˚C	3 min	1 cycle
	END		

Ex:



Run 10 µl PCR products from each reaction in a 2% agarose gel to check size and purity. A light band of about 290bp should be detected as shown on the gel above. Combine amplicons from each reaction to have a volume of 2.6ml PCR1 product for each sample. Perform AMPure XP bead purification (SaSoM Method –XXX-) if purity is not good enough or optimise PCR conditions.

PCR 2

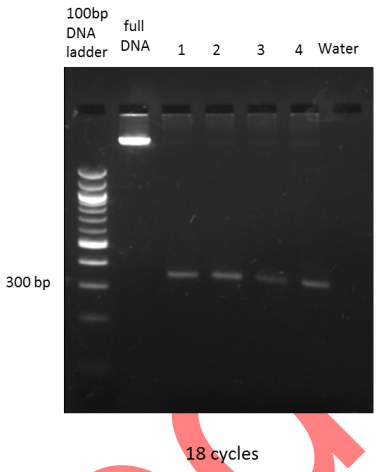
Take **5µl** product from PCR#1 of each sample in 14 PCR tubes and add to the components described in the table below, to get 100 µl reactions. Use the **appropriate primers** (see primer sequences and combinations for PCR2 below). Run PCR protocol.

Components	Targets < 1 kb
dH2O	65µl
5X Herculase buffer	20µl
dNTPs Mix (25 mM each)	1µl
DNA template	**5µl**
Primer 1	2.5µl
Primer 2	2.5µl
Herculase DNA Polymerase	1µl
DMSO	3µl
Total reaction volume	100µl

PCR conditions on G-Storm thermal cycler (Lab 249)

PCR Conditions			
Initial denaturation	95˚C	2 min	1 cycle
Denaturation Annealing Elongation	95˚C 50˚C 72˚C	15 s 20s 30s	18 cycles
Final extension	72˚C	3 min	1 cycle
	END		

Ex:



Check size of PCR products by running 10 µl per lane in a 2% agarose gel. A band of about 350 bp, and similar intensity as on the gel above should be detected.

Purify PCR product from each sample using AMPure XP purification kit (SaSoM Method –XXX)

Proceed to quality control using (i) QubiT (SaSoM Method –079) followed by (ii) Bioanalyzer with High sensitivity DNA kit (SaSoM Method –XXX).

v2Adaptor primers for primary PCR of sgRNA sequences

Primers PCR1	Primer Sequences	Purification	Synthesis scale (µM)
v2Adaptor_ F(Forward)	5'- AATGGACTATCATATGCTTACCGTAACT TGAAAGTATTTCG -3'	HPLC (High performance Liquid chromatography)	0.05
v2Adaptor_ R (Reverse)	5'- TCTACTATTCTTTCCCCTGCACTGTTGT GGGCGATGTGCGCTCTG -3'	HPLC	0.05

Illumina primers for secondary PCR of sgRNA sequences

Primers PCR2	Primer Sequences	Purification	Synthesis scale (µM)
Illumina F1	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTTAAGTAGAGTCTTGTGGAAAGGACGAAACACCG-3'	HPLC	0.05
Illumina F2	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTATACACGATCTCTTGTGGAAAGGACGAAACACCG-3'	HPLC	0.05
Illumina F3	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTGATCGCGCGGTTCTTGTGGAAAGGACGAAACACCG-3	HPLC	0.05
Illumina F4	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTCGATCATGATCGTCTTGTGGAAAGGACGAAACACCG-3'	HPLC	0.05
Illumina R1	5'-CAAGCAGAAGACGGCATACGAGATAAGTAGAGGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTTTCTACTATTCTTTCCCCTGCACTGT-3'	HPLC	0.05
Illumina R2	5'-CAAGCAGAAGACGGCATACGAGATACACGATCGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTATTCTACTATTCTTTCCCCTGCACTGT-3'	HPLC	0.05
Illumina R3	5'-CAAGCAGAAGACGGCATACGAGATCGCGCGGTGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTGATTCTACTATTCTTTCCCCTGCACTGT- 3	HPLC	0.05
Illumina R4	5'-CAAGCAGAAGACGGCATACGAGATCATGATCGGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTCGATTCTACTATTCTTTCCCCTGCACTGT-3'	HPLC	0.05

Combination of Illumina primers for secondary PCR of sgRNA sequences

	Sample	Forward primer	Reverse primer
1	Replicate 1: **Baseline**	Illumina **F1**	Illumina **R1**
2	Replicate 1: **Day 14 Control**	Illumina **F1**	Illumina **R2**
3	Replicate 1: **Day 14 Gemcitabine**	Illumina **F1**	Illumina **R3**
4	Replicate 1: **Day 14 Acelarin S**	Illumina **F1**	Illumina **R4**
5	Replicate 1: **Day 21 Control**	Illumina **F2**	Illumina **R1**
6	Replicate 1: **Day 21 Gemcitabine**	Illumina **F2**	Illumina **R2**
7	Replicate 1: **Day 21 Acelarin S**	Illumina **F2**	Illumina **R3**
8	Replicate 2: **Baseline**	Illumina **F2**	Illumina **R4**
9	Replicate 2: **Day 14 Control**	Illumina **F3**	Illumina **R1**
10	Replicate 2: **Day 14 Gemcitabine**	Illumina **F3**	Illumina **R2**
11	Replicate 2: **Day 14 Acelarin S**	Illumina **F3**	Illumina **R3**
12	Replicate 2: **Day 21 Control**	Illumina **F3**	Illumina **R4**
13	Replicate 2: **Day 14 Gemcitabine**	Illumina **F4**	Illumina **R1**
14	Replicate 2: **Day 14 Acelarin S**	Illumina **F4**	Illumina **R2**

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.

7.0 Training -

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.

SASoM/METHOD/080- PCR for GeCKOv2 library preparation for Next generation sequencing.

SASoM/METHOD/081-sgRNA Target Guide Sequence Cloning into pLentiCRISPRv2.

SASoM/METHOD/082-AMPure PCR purification for GeCKOv2 NGS.

SASoM/METHOD/083-Quality control of GeCKOv2 for NGS using Agilent Bioanalyzer system.

9.0 Approval and sign off –

Author:		
Name:	Paul Reynolds	
Position:	Principal Investigator	
Signature:		Date: 07/10/2021
Management Approval:		
Name:	Peter Mullen	
Position:	Research Fellow	
Signature:		Date: 28/09/2021
QA release by:		
Name:	Alex MacLellan	
Position:	QA Manager	
Signature:		Date: 28/09/2021

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE