Document Number:	**SASoM/METHOD/081.v4**
Title:	**sgRNA Target Guide Sequence Cloning into pLentiCRISPRv2**
Version:	**v4**
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Effective from:	11/11/2020
Valid to:	11/11/2022

SOP History		
Number	Date	Reason for Change
v1	02/04/2015	Original
v2	06/07/2016	Make SOP more user friendly
V3	06/07/2018	Update
V4	11/11/2020	Update

1.0 Purpose –
This SOP describes the current procedure for sgRNA Target Guide Sequence Cloning into pLentiCRISPRv2 in Laboratory 248 at the St Andrews School of Medicine (SASoM).

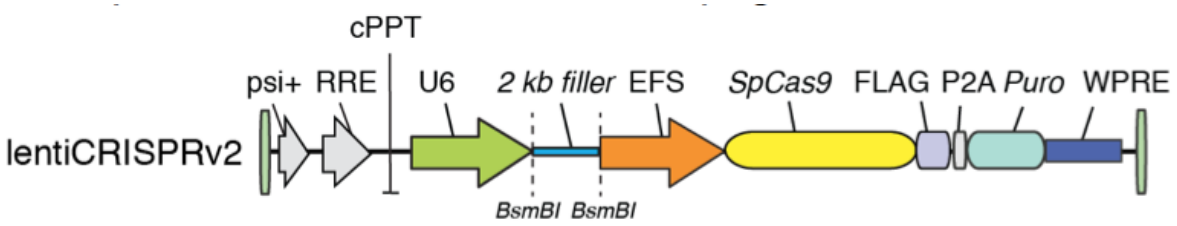
2.0 Scope –
This SOP applies to the staff in the SASoM involved in sgRNA Target Guide Sequence Cloning into pLentiCRISPRv2.

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 documents before performing this procedure.

4.0 Procedure –
pLentiCRISPRv2 (one vector system): This plasmid contains two expression cassettes, hSpCas9 and the chimeric guide RNA. The vector can be digested using *BsmB*I, and a pair of annealed oligos can be cloned into the single guide RNA scaffold, replacing the 2kb filler region. The oligos are designed around the target site

sequence (20bp) and needs to be flanked on the 3' end by a 3bp NGG PAM sequence, as shown on the next page.



Citation: Please reference the following publications for the use of this material.
Sanjana NE, Shalem O, Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. Nature Methods 11: 783-784.
Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343: 83-87.

Target design notes and online resources: For application of Cas9 for site-specific genome editing in eukaryotic cells and organisms, we have computationally identified suitable target sites for the S. pyogenes Cas9 and calculated most likely off-targets within the genome. Please visit http://www.genome-engineering.org to access these Cas9 target design tools. Complete plasmid sequences, protocols, a discussion forum and additional information can be found at the Zhang Lab GeCKO website: http://www.genome-engineering.org/gecko/.

Cloning Steps:

- 1) Digest/AP treat vector pLentiCRISPRv2 DNA
- 2) Design sgRNA oligos
- 3) Anneal oligos
- 4) Ligate oligos and vector
- 5) Grow and purify DNA
- 6) Test digest and Sequence insert
- 7) Make virus

1) Digest and dephosphorylate pLentiCRISPRv2 vector with BsmBI

2 µL vector DNA pLentiCRISPRv2 (2µg)
1 µL FastDigest Esp3I (*BsmBI*) (Fermentas/ Life Technologies FD0454)
2 µL 10X FastDigest buffer
0.5µL CIP (NEB)
0.2 µL 100mM DTT (freshly prepared)
H_2O to 20 µL
Incubate at 37ºC for 30 mins

Prepare 0.8% gel: 0.4g Agarose in 50mL TAE (+1µL ethidium bromide).

2) Gel purify digested plasmid

Run samples on gel at 40V for 45 minutes (171mAmp, 16W).
Upon Esp3I (*BsmBI)* digestion, a 1.8kb filler piece should be present on the gel.
Cut-out and gel purify the larger band ONLY, ~12.8kb. Leave the 1.8kb band (weigh the excised band as this is needed for the gel extraction kit).
Use a QIAquick Gel Extraction Kit and elute in 50 µL of EB (Tris-HCl pH8.5).

3) Design sgRNA oligos of the following form:

For each gene, design a 20mer upstream of a NGG PAM sequence (http://crispr.mit.edu/)

Forward Primer: Add a "CACCG" before 20mer
Reverse Primer: Add a "AAAC" before 20mer and a "C" after 20 mer (see diagram)

Order the oligos at 50nmol yield, 5'phosphorylated and desalted.

4) Anneal Oligos:

Resuspend oligos at 1nmol/ µL

Annealing solution
1 µL of diluted forward oligo (1:10 dilution of stock= 0.1nmol/µL)
1 µL of diluted reverse oligo (1:10 dilution of stock= 0.1nmol/µL)
5 µL of 10x NEB buffer 2 (or 10X T4 ligation NEB buffer)
43 µL ddH2O
Total 50 µL containing 0.2nmol oligos

Put the annealing reaction in a thermocycler using the following parameters:
95ºC 5 min and then ramp down to 25ºC at 5ºC/min

or could use the following:

Incubate 5 min at 95^{0}C. Incubate 10 min at 70^{0}C (in water bath or 1L H_2O) and allow the water to cool to RT.

5) Ligate Oligos and Vector:

Use Roche Rapid Ligation Kit
5 µL of water
1 µL of annealed oligos (ctl no oligo and dilutions 1:10, 1:100, 1:1000)
2 µL of digested gel purified pLentiCRISPRv2 vector (approx. 50ng)
2 µL 5x DNA dilution buffer
10 µL 2x ligation buffer
1 µL ligase
(Total 21 µL)

Incubate at 16ºC overnight.

Vector is 12.8 kb, which means that 50ng is approximately 0.006 pmol.
Annealed oligos are 0.2 nmol in 50 µL solution which means 4pmol/ µL
A 1:40 dilution of annealed oligos solution should give an optimal ratio for the ligation but perform 3 range of dilution (1:10, 1:100, and 1:1000) to confirm and optimise the reaction.

6) Transformation into STBL3 bacteria or Endura electrocompetent bacteria.

Transform chemically competent STBL3 with ligation mix: (Note: can follow the protocol included in with the STBL3's from Invitrogen up until the plating step).

Thaw STBL3 on ice
Add ligation mix to 50 µL STBL3
Incubate on ice 20 min
Heat shock at 42^{0}C for 30sec
Incubate on ice 10 min
Add transformed bugs to 500 µL LB (no amp)
Shake at 37^{0}C for 60 min
Plate 200 µL on LBamp 10cm plates or LBCarb
Incubate at 37^{0}C overnight

Pick colonies – typically 3 colonies per condition to culture
Grow up in 5 mL of LB (100 µg/mL carbenicillin – or amp) for 15 hours overnight (no longer than 16 hours).
Note: keep bacteria agitated during incubation.

Make glycerol stocks of cultures (750µL bacteria + 250µL 80% glycerol)

Mini-prep DNA (include all steps for endA+ bacteria since STBL3 is endA+ strain) and check the concentration of eluted DNA on Nanodrop.

7) Diagnostic Digest to Verify Inserts

Check which of your colonies carry the correct insert (sgRNA) in pLentiCRISPRv2 by performing a NdeI/ EcoRI double digestion. This should cut the vector into 3 fragments: 10,347bp, 2,299bp and 154bp. The smallest fragment should increase by 20bp when containing an insert.
Run digested DNA on a 2% agarose gel (P2: 60V, 55min) and observe the 154bp / 174bp fragment.

Notes:
*run with hyperladder I and 1kb marker
*image once band is ¾ down the gel, often lower bands are'nt visible at first, shake gel (~20 RPM) for 30-60 minutes in 50 mL TBE + 2µL EtBr)
*re-image, lower bands will be visible

To sequence inserts, use:
pLKO.1 5' (5'- GACTATCATATGCTTACCGT-3').

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 sterile TC techniques before starting any TC work

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-001- DNA electrophoresis using agarose gels

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 –

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Management Approval:		
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