Document Number:	**SASoM/METHOD/046.v5**
Title:	**Cloning and validating a 19mer shRNA hairpin in pLKO.1-puro.**
Version:	**v5**
Author:	**Paul Reynolds**

Effective from:	10/08/2021
Valid to:	09/08/2023

SOP History		
Number	Date	Reason for Change
v1	29/08/2013	Original
v2	10/08/2015	Update
v3	10/08/2017	Update
V4	10/08/2019	Update
V5	10/08/21	Update

1.0 Purpose –
This SOP describes the current procedure for cloning a 19mer shRNA hairpin to pLKO.1puro for use 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 cloning and validating a 19mer shRNA hairpin to pLKO.1-puro.

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 –

Steps:
Digest/CIP treat pLKO.1-puro empty vector
Design shRNA oligos
Anneal oligos
Ligate oligos and vector
Grow and purify DNA
Test digest and Sequence insert
Make virus, infect cells and perform Western blotting.

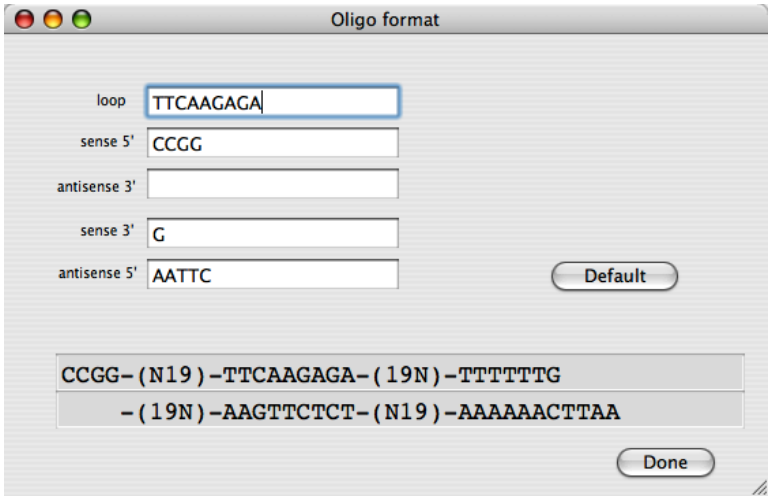
Digest/CIP Treat Vector: pLKO.1-puro

1 μg pLKO.1-puro vector maxiprep DNA
2 μL NEB buffer 1
1 μL AgeI (NEB)
1 μL EcoRI (NEB)
H2O to 20 μL
Add 0.5 μL CIP
Incubate 37^0 1 hr

Run digest plus loading dye in an agarose gel and extract DNA band using a Gel purification kit (Qiagen). Elute in 50 μL of MilliQ water, do NOT follow the elution instructions from the kit. (see protocols for agarose gels).

Design Short-hairpin Oligos:
Find 19-mer sequence to target gene
Tip—Use sequences that are verified in publications and have pictures of western blot knockdown at protein level.
e.g. Use the program psicoligomaker 1.5 from Tyler Jacks lab to design oligos
http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html
Directions below:

For pLKO.1:
Type 19-mer gene-specific sequence in “Target”
Click “Settings”



Change end sequences
loop = TTCAAGAGA
sense 5' = CCGG
antisense 3' =
sense 3' = G
antisense 5' = AATTC
Click "Done"
Click "Make oligos"
The "For Oligo" is your forward oligo
The "Rev Oligo" is your reverse oligo

Order the oligos from Invitrogen, using the following citeria:
Desalted, 50nmol, 5'phosphorylated.

Anneal Oligos:
Centrifuge tubes briefly, 10 sec at 10,000rpm. Resuspend oligos at 1nmol/µL with sterile MilliQ water.
Set up water bath at precisely 70^{0}C. Check with thermometer.

Annealing solution: in an eppendorf tube,
1 µL of diluted forward oligo (1:10 dilution in MilliQ water of stock)
1 µL of diluted reverse oligo (1:10 dilution in MilliQ water of stock)
5 µL of 10x NEB buffer 2
43 µL MilliQ water to a final volume of 50µL.

Incubate 4 min at 95^{0}C, then
Incubate 10 min at precisely 70^{0}C in water bath - use a thermometer (in water bath or 1L beaker of H2O heated by a Bunsen) and allow the water to cool to RT (takes a few hours). Then move to 4^{0}C.

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, CIP treated, gel purified vector
2 μL 5x DNA dilution buffer
10 μL 2x ligation buffer
1 μL ligase

Incubate at RT for 1-24 hrs

Grow and Purify DNA:

Transform DH5α with ligation mix:

Thaw DH5α on ice (subcloning grade from Invitrogen)
Add ligation mix to aliquoted 50 μL DH5α
Incubate on ice 20 min
Heat shock at 42^{0}C for 30sec in heat block
Incubate on ice 10 min
Add transformed bugs to 500 μL LB (no selection)
Shake at 37^{0}C for 60 min
Plate 200 μL on LB /100 μg/mL carbenicillin 10cm bacterial plates
Incubate, inverted at 37^{0}C overnight in bacterial incubator
Pick colonies into culture tubes:
Grow up in 3 mL of LB (100 μg/mL carbenicillin (Sigma))
Mini-prep DNA using kit.

KEEP ALL CULTURES IN FRIDGE (even empty tubes) AND ALL DNA UNTIL CLONES ARE VERIFIED BY DIGESTION AND SEQUENCING.

Test Digest and Sequence Insert:

Test digest

For pLKO.1

7 μL MilliQ water and 1ug of mini-prep DNA
1 μL NEB buffer 1
0.1 μL BSA
1 μL AgeI (NEB)
1 μL SpeI (NEB)

Incubate at 37^{0}C 1 hr
Test on 1% agarose gel (follow agarose gel protocols)

Expected result
For pLKO.1-puro:

Loss of 469 bp band if insert is correct (note: oligos should destroy AgeI site, however if sense 5' oligo starts CCGGT AgeI site is not destroyed and there will be a 469 bp to 528 bp shift if the insert is correct).

Sequencing primer:
pLKO.1-seq: 5' GACTATCATATGCTTACCGT

Use this primer with DNA and follow protocol for sequencing at Dundee sequencing facility for those that pass the digestion test.

If sequence correct, make a glycerol stock from contents of bacterial culture:
750 μL of bacterial culture
250 μL of 80% sterile glycerol
in a cryovial. Mix and store at -80^{0}C in Reynolds Lab glycerol stock box.

AT THIS STAGE YOU MAY DISCARD OTHER, NON-VALIDATED CLONE MATERIALS.

Culture validated bacterial stock to make maxiprep DNA (follow maxiprep DNA protocol).

Use this DNA to make virus and infect recipient cells (follow lentivirus protocols).

Only when protein of interest is knocked down in cells, as assessed by western blotting, can the shRNA be considered as validated.

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 100% ethanol or 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/04 - Culture of primary and established cell lines
RA/GM/001 - Working with Category 2 (Cat 2) viruses

8.2 SOPs -
SASoM/METHOD/047 – siRNA transfection of mammalian cells

SASoM/METHOD/048 – DNA Sequencing at the University of Dundee

SASoM/METHOD/050 Biorad Mini Western Blotting

SASoM/METHOD/052 Agarose Gels

9.0 Approval and sign off –

Author:

Name: Paul Reynolds

Position: Principal Investigator

Signature:  Date: 07/10/2021

Management Approval:

Name: Peter Mullen

Position: SOP Administrator

Signature:  Date: 28/09/2021

QA release by:

Name: John O' Connor

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