Winnie Chang

Construct pLKO.1 shRNA plasmids for gene knockdown

1. Vector backbone

(1) pLKO.1-TRC cloning vector -Puro (Addgene, #10878)

(2) pLKO.1-tRFP657 (derived from (1) but exchange Puro into tRFP657)

2. Design shRNA oligos for pLKO.1

Determine 21-mer target in your gene using **Predesigned shRNA** (MERCK)

https://www.sigmaaldrich.com/JP/en/semi-configurators/shrna?activeLink=productSearch

-> Search Genes/Clones: key in your interest gene symbol -> search

-> Select shRNA Clones: choose the species and click details. Ex:

TRCN0000148112 | Validated | SETDB1 | 9869 | NM_012432 | Human | CDS | **Details**

Target Sequence: GCTCAGATGATAACTTCTGTA
Gene Synonym: ESET, H3-K9-HMTase4, KG1T, KMT1E, TDRD21

Cell Type: A549
Method: SYBR
Knockdown: 95%
Version: 1

Choose validated knockdown efficiency > 90%

Copy Target Sequence as 21-mer as sense, and use complementary sequences as antisense

3. Order Oligos Compatible with pLKO.1

To generate oligos for cloning into pLKO.1, insert your sense and antisense sequences from step 2 into the oligos below. Do not change the ends; these bases are important for cloning the oligos into the pLKO.1 TRC-cloning vector.

Forward oligo:

5’ CCGGCCC – **21bp sense** – CTCGAG – **21bp antisense** – TTTTTGGAAAT 3’

Reverse oligo:

5’ AATTATTTCCAAAAA – **21bp sense** – CTCGAG – **21bp antisense** – GGG 3’

Order oligos with 100 µM DDW

Ex: for human SETDB1

Forward oligo:

CCGGCCC***GCTCAGATGATAACTTCTGTA***CTCGAG***TACAGAAGTTATCATCTGAGC***TTTTTGGAAAT

Reverse oligo:

AATTATTTCCAAAAA***GCTCAGATGATAACTTCTGTA***CTCGAG***TACAGAAGTTATCATCTGAGC***GGG

4. Cloning Oligos into pLKO.1

The pLKO.1-TRC cloning vector contains a 1.9 kb stuffer that is released upon digestion with EcoRI and AgeI

(1) Digesting pLKO.1 vector

Vector (6 μg)	6 μl
10x rCutSmart	6 μl
EcoRI	1 μl
AgeI	1 μl
DDW	46 μl
Total	60 μl

Incubate at 37°C for 2 hours and run agarose gel

(2) Annealing oligos

Forward oligo (100 μM)	1 μl
Reverse oligo (100 μM)	1 μl
10x NEB buffer 2	5 μl
DDW	43μl
Total	50 μl

95°C 4 min

70°C 10 min

Take out the heat block and cool it down to room temperature (at least 1 hour)

(3) Run 0.8% agarose gel (100V, 30 min) with step 4-1 digested vectors

Expected fragment size: 1.9 kb (stuffer), **7 kb (cut vector)**

-> Cut 7 kb digested vector and do gel extraction

-> Elute DNA with 25 μl DDW

(4) Ligation

*10x dilution for annealed oligos: 5 μl mixture (from step 4-2) + 45 μl DDW

10x diluted oligos	3 μl
Purified vector (step 4-3)	1 μl
2x mighty ligation mix	4 μl
Total	8 μl

Incubate at 16°C for 1-3 hours

(5) Transformation (DH5α)

-> Take 3 μl of ligation product (step 4-4) into 25 μl of DH5α

-> on ice 30 min -> heat shock 42ºC for 45 sec -> on ice 3 min -> add 80 μl SOC

-> all for plating with Amp plate

(6) Mini-culture

Pick up 4 colonies each group for mini-culture (2 ml LB+2 μl Amp)

(7) Mini-prep

Dissolve DNA with 30 μl TE

(8) Enzyme digestion check

Mini-prep DNA	2 μl
10x buffer M	2 μl
PvuII	0.3 μl
DDW	15.7 μl
Total	20 μl

(9) Run 1% agarose gel (100V, 20 min)

Correct expected fragment size: 3712, 2513, 776 bp

Negative control of original vector cutting size: 2810, 2604, 2513, 776, 198 bp

(10) Select correct plasmids for sequencing

<u>*Sequencing method: dGTP</u>

Select Premix1 sequencing and note dGTP 使用 to 特記事項欄

【Premix1 シングル解析】¥2,000 / サンプル

テンプレート DNA と Primer を混合したサンプルをご提供いただきます。

シーケンサーにて 2 時間の泳動を行い、ご提供の base 数は 1000bp 程度になります。

Web サイトより Premix1 シングル解析をご選択いただき、「特記事項欄」に、「dGTP 使用」と予めご記入いただきますと、シーケンス反応時に弊社にて dGTP 試薬を使用して解析を実施します。