Manual for the Establishment of LacO/IPTG Controlled shRNA Inducible Cell System

Introduction

Inducible approaches are necessary for the functional and biochemical characterization of essential or survival genes. In addition, we have been observing that many shRNAs could not be packaged into VSV-G pseudotyped lentiviruses in 293T producer cells or the lentivirus titers are very low. This could be due to one of the following reasons: (i) Effective siRNA targets important genes such as essential, survival genes of packaging cells; (ii) Effective siRNAs may target cellular genes that are required for lentivirus assembly or budding; (iii) Other reasons. However, these possibilities are not mutually exclusive. Therefore, inducible expression of shRNA can provide significant advantage for the study of essential or survival genes.

The shRNA expression systems in cells are most often controlled by RNA polymerase III (pol III) promoters. In this system, transcription initiation site of RNA pol III promoter is well defined, and transcription stops when polymerases encounter consecutive 4-5 Ts and terminates at the second U (T on DNA template). Thus, pol III transcripts result in uniform RNA molecules containing defined 5' and 3' ends. During the last few years, several inducible systems have been developed to control expression of shRNAs by using U6p promoter. The RNAi Core has developed a LacO/IPTG shRNA inducible system in which 3 copies of LacI operator sequences were introduced into U6p-based promoter to regulate the expression of shRNA. The plasmid map of this inducible vector (pLAS1w.3xLacO) is depicted in **Figure 1**.

In this manual, we describe how to use this vector to establish an inducible cell line. The manual includes the following guidelines:

I. Cloning shRNA sequence of interest into pLAS1w.3xLacO
II. Production of VSV-G pseudotyped lentivirus
III. Titration of virus titer
IV. Knockdown measurement of inducible shRNA
V. Optimization of assay condition
VI. Figures
VII. Appendix (Materials used and Links)

Guidelines

I. Cloning shRNA sequence of interest into pLAS1.3xLacO

This section describes the protocol on how to clone a desired shRNA into inducible vector, pLAS1w.3xLacO.

The cloning vector of pLAS1w.3xLacO contains a 1.9 kb fragment of stuffer sequence inserted in between the shRNA cloning sites of *Bfu*AI and *Eco*RI. After double cutting by *Bfu*AI and *Eco*RI, this allows one to monitor the cutting efficiency of the vector by observing the release of 1.9 kb fragment from the vector.

Experimental procedure:

A. Preparation of cloning vector:

1. Incubate 3-5 ug of pLAS1w.3xLacO with 10 units of *Eco*RI in NEB buffer #3 with a volume of 100 ul for O/N at 37^{O}C; on second day, add 10 units of *Bfu*AI into *Eco*RI-digested mixture and incubate it at 50^{O}C for at least two hours. A complete digestion of this vector was observed if only 8kb and 1.9 kb fragments were seen on the gel. The digestion pattern was provided in **Figure 2** for your reference.

2. Separate the cut DNA by 0.8% agarose gel electrophoresis, purify the 8 kb fragments from the gel with appropriate method developed in your lab.

3. Elute/dissolve DNA with appropriate volume (30-50ng/ul in 0.1xTE buffer is recommended).

B. Preparation of insert (annealing method):

1. Design sense (tail with **CCGG** sequence at the 5' end) and antisense (tail with **AATT** sequence at the 5' end) shRNA oligonucleotide as follows:

   Given cgcatacgacgattctgtgat as the target sequence, the sequences of sense (up-strand sequence of following example) and antisense (low-strand sequence of following example) oligonucleotide would be as follows:

   target sequence — anti-sense sequence

   CCGGcgcatacgacgattctgtgatctcgagatcacagaatcgtcgtatgcgttttt

   gcgtatgctgctaagacactagagctctagtgtcttagcagcatacgcaaaaaTTAA

   ctcgag: loop sequence of shRNA

   Please note that the design is in concert with TRC's shRNA library design.

2. Order oligonucleotides with 200 nmole scale purified by OPC or PAGE purification (oligonucleotide produced by Mission Biotech recommended).

3. Dissolve oligonucleotides into 100 µM with autoclaved distillated water.

4. Prepare 10X annealing buffer:

   1M K-acetate
   0.3M HEPES-KOH pH7.4
   20 mM Mg-acetate

5. Set up annealing mixture:

Sense oligo	9 µl
Antisense oligo	9 µl
10X annealing buffer	2 µl

6. Anneal mixture by PCR machine using the following parameters:
   95°C, 78°C, 74°C, 70°C, 67°C, 63°C, 60°C, 56°C, 63°C, 60°C, 56°C, 53°C, 50°C, 48°C, 46°C, 44°C, 42°C, 40°C, 39°C, 37°C, 36°C, 35°C, 34°C, 33°C, 32°C,31°C ----------5 min in each step followed by 30°C, 28°C, 26°C, 24°C, 22°C, 20°C----------10 min in each step,
   Finally, hold at 4°C

   Then dilute the annealing product 500x with ddH_2O.

7. Set up ligation mixture:

RE-cut pLAS1w.3LacO	50-100 ng
Annealed oligonucleotides	1-2 ul
Takara ligation mix (#6023)	equal to the total volume of vector and insert

   Incubate ligation mix at RT, 1 hour. *(Note) Total ligation mix volume should not exceed 10% of the competent cell volume.*

8. Take all of the ligation mix for transformation (Stbl3 strain is recommended for lentiviral vector transformation).

9. Pick several colonies, culture them in TB medium O/N, and perform mini-prep according to the manufacturer's manual.

10. Check shRNA sequence by the following primer:

    5'-ATTTCTTGGGTAGTTTGCAG-3' (For sequencing forward strand of shRNA)

II. Production of VSV-G pseudotyped lentivirus

The production of LacO controlled shRNA lentivirus is the same as 3-plasmid system for VSV-G pseudotyped lentivirus production. This lentiviral production link will give you the detailed information on how to produce lentivirus in 6 cm dish or 96-well plate.

III. Titration of virus titer

The virus titer determined in A549 is sufficient for the subsequent experiments such as establishing stable cell line if the virus harboring drug selection marker for selecting transduced cells. However, you still may determine the virus titer using the cell line that you intend to be established. The following links will give you the detailed information on how to titrate lentivirus by RIU, or CFU.

IV. Knockdown measurement of inducible shRNA

An effective shRNA (TRCN0000003756) targeting *P53* gene was introduced into pLAS1w.3xLacO vector according to the method described in the Section of *Cloning shRNA sequence of interest into pLAS1.3xLacO*. Lentivirus expressing P53 inducible shRNA was produced and titrated. The resulting virus was tested its tightness in A549 cells by monitoring the remaining amount of P53 protein before and after induction with IPTG. Furthermore, the responsibility of this vector to IPTG induction was compared to a pLKO_TRC005-based constitutive vector expressing the same shRNA. The following protocol serves as a general guideline for your reference.

The guidelines are outline as following table and detailed protocol is described in the experimental procedure as follows:

	Task to be done
Day 1	Seed cell
Day 2	1. Refresh media containing inducible lentivirus and polybrene 2. Centrifugation to promote infection (optional)
Day 3	Change media containing puromycin
Day 4	Seed cell for constitutive control
Day 5	Change media containing IPTG/ Infect day 4 cells with constitutive controls
Day 6	Change media containing puromycin in constitutive control cell
Day 9	Assay

All procedures should be carried out in accordance with biosafety requirements of the host institution.

Experimental Procedure

[OPTIONAL]

(Red typefaces are procedures for constitutive control experiment)

1. Day1: Seed A549 cell at a density of 20-30% confluence (0.75 ml/well) in a 12-well plate. (Increasing the seeding media volume may cause the cells uneven distribution on cultural plate). Incubate the cell overnight (37°C, 5%CO_2).
2. Day2: Remove cell media, and then add 1 ml of media containing 8 ug/ml polybrene and pLAS1.3xLacO-shP53 virus (MOI equals to 3). Centrifuge the plate for 30 minutes, 1200 g, 37˚C. Incubate the cell overnight (37°C, 5%CO_2).
3. Day3: Remove infection media and replace with 2 ml media containing 2 ug/ml puromycin. Incubate the cell for 2 days (37°C, 5%CO_2).
4. Day4: For constitutive control, seed A549 cell at a density of 20-30% confluence (0.75 ml/well) in a 12-well plate. Incubate the cell overnight (37°C, 5%CO_2).
5. Day 5: Remove the puromycin media, then passage and seed the cell onto 6-well plate with 2 ml media containing 500 uM IPTG. The IPTG media was added directly during subculture.
6. Day 5: Remove cell media of item 4, and then add 1 ml of media containing 8 ug/ml polybrene and pLKO.1-shP53 or pLKO.1-shScramble virus (MOI 3), 6 wells for each virus. Centrifuge the plate for 30 minutes, 1200 g, 37˚C.
7. Day 6: Remove infection media of item 6 and replace with 1 ml media containing 2 ug/ml puromycin and 500 uM IPTG. Incubate the cell for 2 days (37°C, 5%CO_2).
8. Day 9: Lyse the cells for Western analysis.
9. Results of Western data are shown in **Figure 3.**

V. Optimization of LacO inducible lentivector-shRNA assay

To gain a better result, the following conditions should be optimized:

- Amount of lentivirus added (MOI)
- Puromycin concentration
- IPTG concentration
- Centrifugation time
- Time course

This link should give you some idea towards beginning your own optimization.

Cell Seeding

- It is advised that the cell shall be seeded on the scale of 24-well to 6-well plates; therefore, spin infection could be performed after adding the virus

- An even distribution of cell on the plate surface is important to ensure that every cell has the same probability to get infected by the viruses.

Virus Infection

- For suspension cell line, the cell seeding step could be skipped. Mix the cell with media containing virus and polybrene, then centrifuge to improve infection efficiency

- The final polybrene concentration for the infection media is 8 μg/ml, and if polybrene is toxic to the cell line, it may be substituted with protamine sulfate.

- For centrifugation, spin cell using 1200 g, 15-90-min (depends on cell line), 37 °C. *If using 6-well plates, it should be rotated for turns to ensure even infection and avoid certain cell area from drying.*

- If your institution does not have centrifuge suitable for lentivirus spin infection, then, higher MOI or longer incubation period after infection (without polybrene) could be carried out to increase the number of cell to be infected.

- If any constitutive expression based lentivirus-shRNA were used during the course of the experiment, then it is highly advised that the infection of constitutive virus shall be done at the same timing of adding IPTG on the inducible cell. This ensures the same number of days in which the shRNA expressed in the cell.

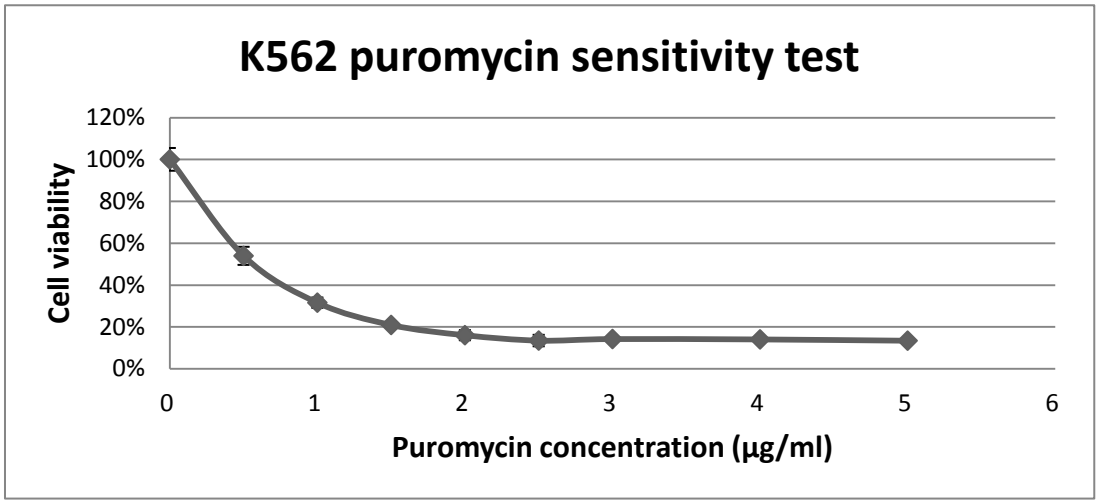
Puromycin

Puromycin concentration and incubation time should be optimized for each cell line, typical concentration range from 1-5 μg/mL and incubated for at least 48 hours.

To optimize the puromycin treatment, the following steps could be done:

Day 1	Seed 25% confluence cells onto 3 96-well plates.
Day 2	1. Set up serial dilution of puromycin ranged 0-10μg/mL in the cell growth media. 2. Add equal volume of puromycin media into each well, therefore the final puromycin concentration ranged from 0-5μg/ml.
Day 4	1. Replace puromycin media with 100μl phenol red-free DMEM containing 10% MTS (V/V). 2. Incubate the plate for 40-50 minutes (37°C, 5%CO_2). 3. Read the absorbance at 490 nm using a 96-well plate reader. 4. Determine the relative viability by comparison with the no puromycin wells (setting as 100%).
Day 5	If 2 days puromycin selection is insufficient to kill most of the cell, repeat day 4 step (Optional).
Day 6	If 3 days puromycin selection is insufficient to kill most of the cell, repeat day 4 step.

Example,



K562 cell line was given different concentrations of puromycin 0-5 ug/ml for 2 days, then MTS assay. From this result, 2.5 ug/ml was taken for selection on further experiments.

Or, to roughly estimate the puromycin concentration needed, the following steps could be done:

Day 1	Seed 25% confluence cell onto 3 24-well plates.
Day 2	1. Set up serial dilution of puromycin ranged 0-5μg/mL in the cell growth media. 2. Replace media with puromycin containing media.
Day 4	Observe under light microscope which concentration resulted in ~95% cell death.
Day 5	(Optional) If 2 days puromycin selection is insufficient to kill most of the cell, repeat day 4 step.
Day 6	(Optional) If 3 days puromycin selection is insufficient to kill most of the cell, repeat day 4 step.

IPTG

IPTG concentration and incubation time should be optimized for each cell line, typical concentration range from 0.1-0.5 mM and incubated for at least 72-96 hours, and the IPTG-containing media must be refreshed every 48-72 hours. The following recommendation are general guidelines only, and should be optimized for a given cell line, target gene, and assay.

Post infection assay	IPTG incubation time
mRNA knockdown (qPCR)	3+ days
Protein knockdown (western)	4+ days
Phenotypic assay	5+ days

Recovery phase

The expression of shRNA in this system is reversible. Upon removal of IPTG from the media, the target gene shRNA expression could be re-suppressed, allowing the target gene expression to be recovered. In order to optimize the recovery phase, it is highly advised that the cell culture is washed at least 3 times with PBS, trypsinized (for adherent cell), and centrifuged for 300 g, 3 minutes then remove supernatant to allow complete removal of IPTG from the culture media. The following recommendation are general guidelines only, and should be optimized for a given cell line, target gene, and assay.

Assay	Recovery time
mRNA expression (qPCR)	3+ days
Protein expression (western)	4+ days
Phenotypic assay	5+ days

VI. Figure1

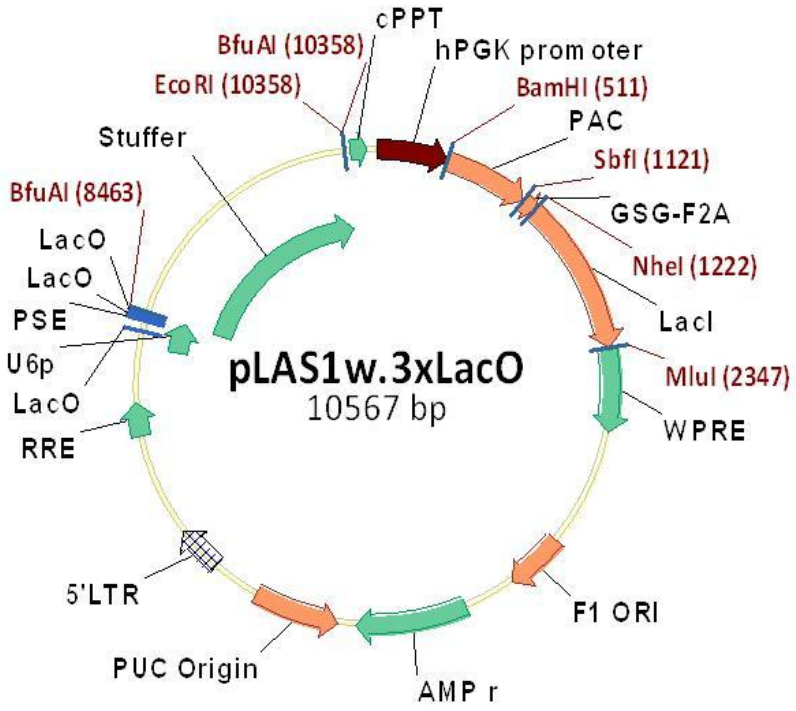


Figure 1. Map of pLAS1w.3xLacO. pLAS1w.3xLacO was derived from pLKO_TRC005 with the following modifications:

1. Three portions of sequences on U6p promoter were replaced with three copies of LacI operator sequence which are labeled with LacO on the map;
2. A 1.9 kb fragment of stuffer sequence was inserted into shRNA cloning sites, *Bfu*AI and *Bfu*AI/*Eco*RI. This allows one to monitor the cutting efficiency of the vector by observing the release of the 1.9 kb fragment from the vector after digesting it with *Eco*RI and *Bfu*AI;
3. ORF of *LacI* repressor was introduced into the downstream of *PAC* gene by fusing it with GSG-F2A sequence to link *PAC* ORF. This fusion results in maintaining the same reading frame from *PAC* to *LacI*.

Location of Features:

- PAC (Puromycin acetyltranferase) : nt519-1115
- GSG-F2A : nt 1122-1220
- LacI (Lac Inhibitor) : nt1227-2345
- LacO (Lac Operon) : nt 8322- 8342; 8417-8436, 8443-8462.

Note:

To remove the 1.9 kb stuffer completely, a double digestion of *Bfu*AI and *Eco*RI is recommended. More detailed information is written on **Figure 2**.

Figure2

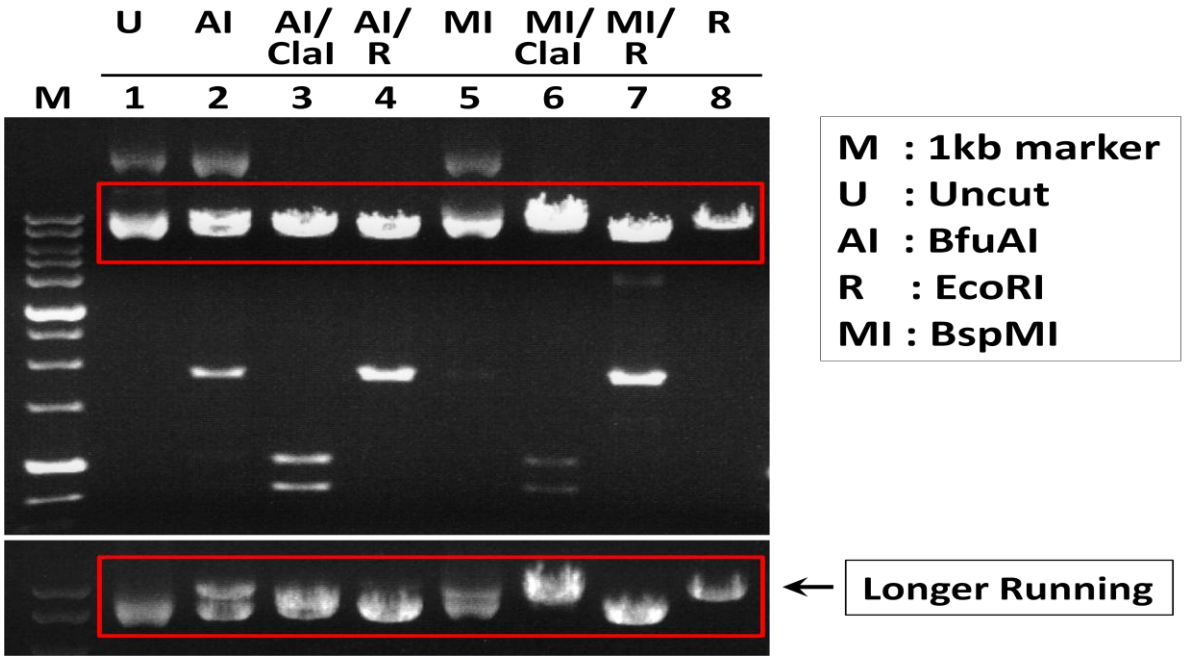


Figure 2. A double digestion of *Bfu*AI/*Bsp*MI plus *Eco*RI efficiently restricts the plasmid of pLAS1.3xLacO for shRNA cloning. Each lane contained 200 ng of pLAS1.3xLacO (Digestion was performed in a volume of 12ul with restriction enzymes less than 0.8ul). Although the 1.9 kb fragment of the plasmid is flanked by 2 *Bsp*MI and *Bfu*AI cutting site, our results showed that a single RE digestion of *Bsp*MI or *Bfu*AI could not completely remove the fragment (**Lane 2 & 5**). A double digestion of *Bsp*MI or *Bfu*AI with *Cla*I is not recommended due to different buffer requirement is needed to achieve optimal result (**Lane 3 & 6**). A double digestion of *Eco*RI and *Bfu*AI is recommended for preparing this vector. The conditions are as follows: DNA first digests with *Eco*RI for appropriate time to ensure the plasmid DNA is linearilized, then add *Bfu*AI directly to *Eco*RI-digested mixture and incubate at 50^{o}C for at least two hrs (**Lane 4**). A double digestion of *Bsp*MI and *Eco*RI could also achieve the same efficiency in fragment removal as its isoschizomer applied (**Lane 7**).

Figure3

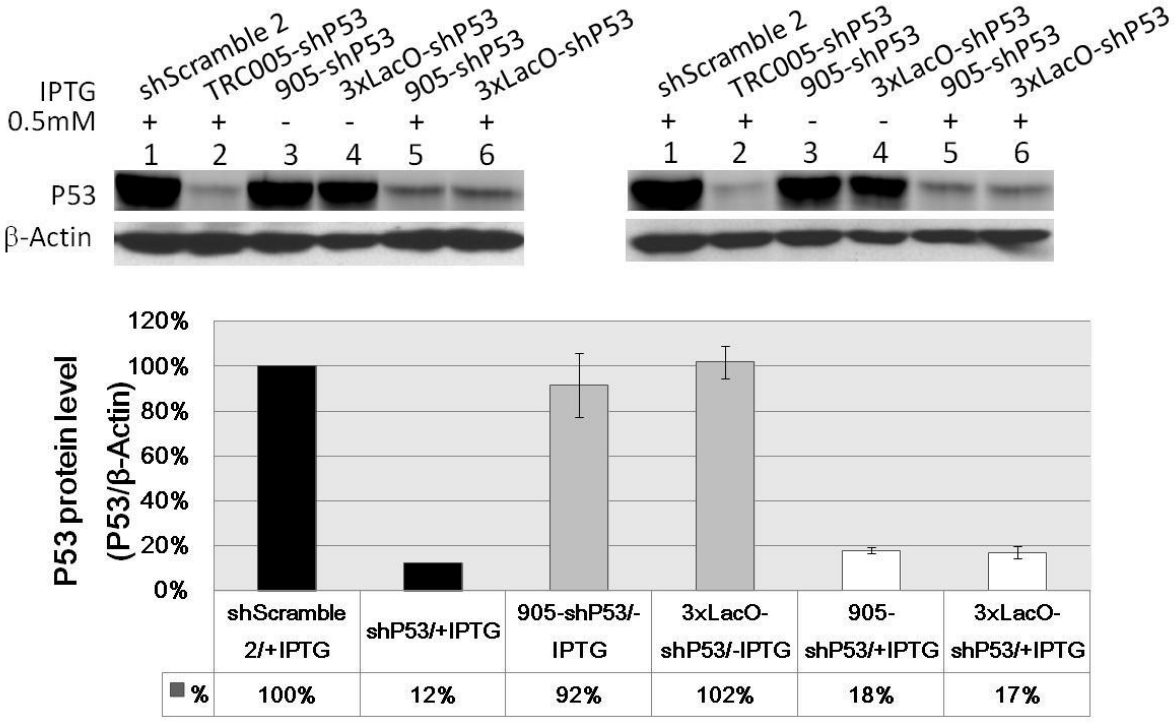


Figure 3. Tightness and responsibility of pLAS1.3xLacO vector. The results of lower panel were obtained from two independent experiments shown in upper panel. 905-shP53 was derived from TRC vector called pTRC-905, which also contains 3 copies of LacO operator but one of the LacO operator location is different from pLAs1.3xLacO. Western blotting lysate preparation. Cell was washed with cold PBS, scraped, then centrifuged to pellet the cell, supernatant was removed and appropriate volume of CelLysis M reagent (Sigma #C2978) supplemented with protease inhibitor cocktail (Roche #04693132001) was added, Incubate at RT for 30 mins then centrifuge for 15 mins, 13000 rpm. Next, lysate was transferred into pre-cooled 1.5 ml microcentrifuge tube for further western analysis.

VII. Appendix

Materials

Polybrene (Hexadimethrine bromide; Sigma H9268) or Protamine Sulfate (Sigma #4026)
Puromycin Dihydrochloride (Sigma #P8833)
IPTG (MDBio.Inc #101-357-93-1)
Restriction Enzymes : *Bfu*AI (NEB #R0701); *Bsp*MI (NEB #R0502); *Cla*I (NEB # R0197)
*Eco*RI (NEB R0101)
Takara ligation mix (#6023)
Cellytic M Cell Lysis Reagent (Sigma #C2978)
cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche #04693132001)

Links

Lentivirus production protocol link
(http://rnai.genmed.sinica.edu.tw/file/protocol/2_LentivirusProductionV3.pdf)

Estimation of lentivirus titer by RIU link
(http://rnai.genmed.sinica.edu.tw/file/protocol/4_1_EstimationLentivirusTiterRIUV1.pdf)

Estimation of lentivirus titer by CFU link
(http://rnai.genmed.sinica.edu.tw/file/protocol/4_2_EstimationLentivirusTiterCFUV1.pdf)

Optimization of lentivector-shRNA assay link
(http://rnai.genmed.sinica.edu.tw/file/protocol/TRC_cell_linestransduced_details.pdf)