Manual for the Establishment of Tetracycline 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 tetracycline(Tet) shRNA inducible system in which 8 copies of TetO operator sequences were introduced into U6p-based promoter to regulate the expression of shRNA. The plasmid map of this inducible vector 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.8xTetO
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 pLAS1w.8xTetO

The protocol of cloning a desired shRNA into inducible vector, pLAS1w.8xTetO is the same as "Protocol for shRNA construction-II: annealing method". The cloning vector of pLAS1w.8xTetO contains a 1.9 kb fragment of stuffer sequence inserted in between the shRNA cloning sites of *Age*I and *Eco*RI. After double cutting by *Age*I 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.

II. Production of VSV-G pseudotyped lentivirus

The production of TetO 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 *and* Scramble (ASN0000000003) as shRNA control were introduced into pLAS1w.TetR, pLAS1w.3xTetO and pLAS1w.8xTetO plasmids respectively. Lentiviruses expressing P53 inducible shRNA or control were produced and titrated. The resulting viruses were tested its tightness in A549 cells by monitoring the

remaining amount of P53 protein before and after induction with doxycycline(Dox). The following protocol serves as a general guideline for your reference.

The guidelines are outline as following table:

	Task to be done
Day -1	Seed cell
Day 0	1. Refresh media containing inducible lentivirus and polybrene 2. Centrifugation to promote infection (optional)
Day 1	Change media containing puromycin
Day 2	Lyse the cells 2 days after infection for Western analysis
Day 4	Lyse the cells 4 days after infection for Western analysis
Day 5	Change media containing Dox
Day 6	Lyse the cells 6 days after infection for Western analysis
Day 8	Lyse the cells 8 days after infection with or without Dox treatment for Western analysis
Day 9	Western blot assay

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

V. Optimization of TetO inducible lentivector-shRNA assay

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

- Amount of lentivirus added (MOI)
- Puromycin concentration
- Doxycycline 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.

Puromycin

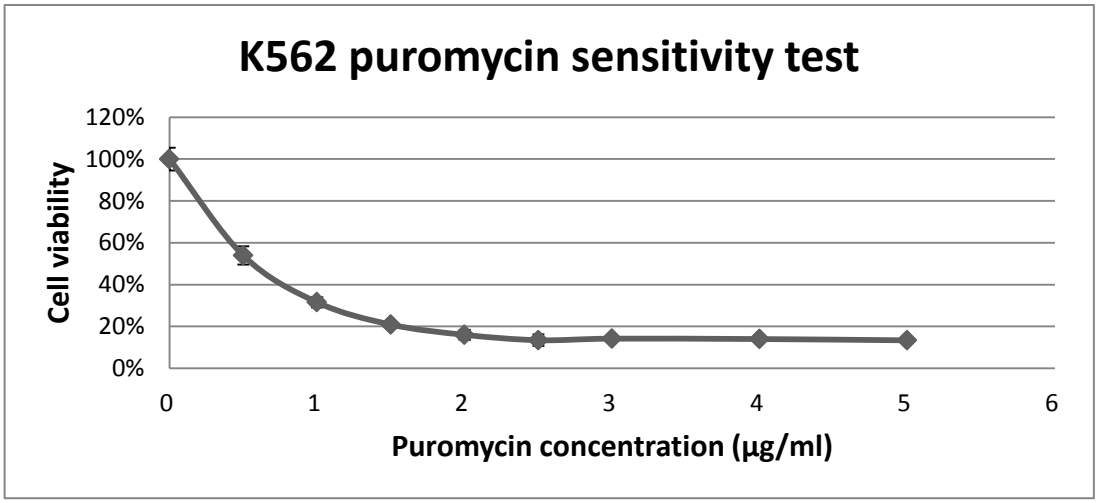
Puromycin concentration and incubation time should be optimized for each cell line, typical concentration range from 1-5 μg/mL and incubated for at least 48 hours.

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. 2. Incubate the plate for 40-50 minutes (37°C, 5%CO2). 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	(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.

Example,



K562 cell line was given different concentrations of Puromycin 0-5 μg/ml for 2 days, then MTS assay. From this result, 2.5 μg/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.

Doxycycline (Dox)

Doxycycline, a tetracycline derivative, is currently the most preferable effector substance for Tet-inducible system. Dox has been shown to have a longer half-life than tetracycline (48 hours vs. 24 hours, respectively). Dox concentration and incubation time should be optimized for each cell line, typical concentration range from 0.1-2 μg/ml. Cell should be incubated in Dox-containing media for at least 72-96 hours, and the Dox-containing media

must be refreshed every 48 hours. The following recommendation are general guidelines only, and should be optimized for a given cell line, target gene, and assay.

Post infection assay	Dox 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 Dox from the media, the 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 Dox 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

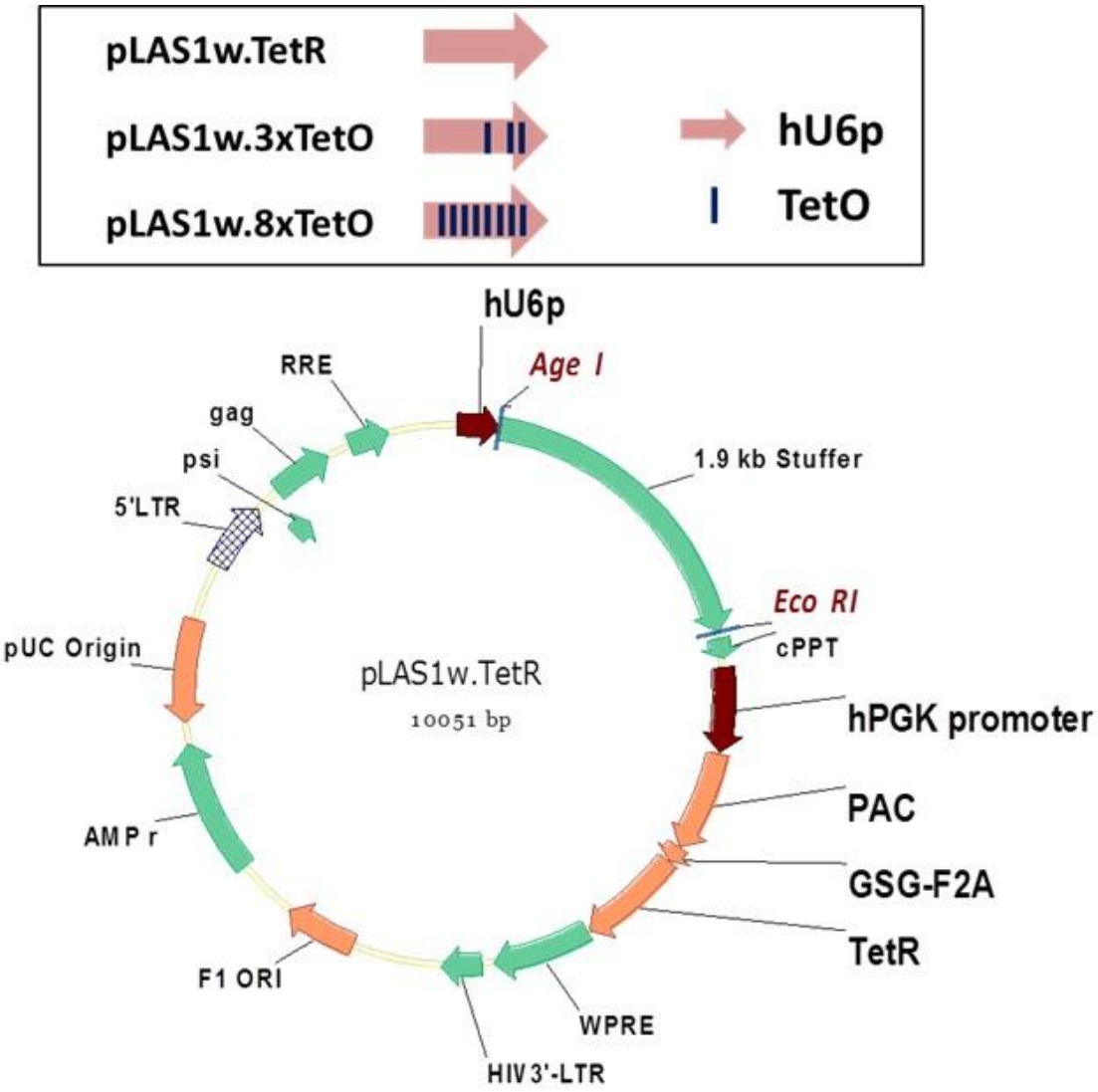


Figure 1. Map of pLAS1w.TetR, pLAS1w.3xTetO and pLAS1w.8xTetO. A 1.9 kb fragment of stuffer sequence was inserted into shRNA cloning sites of *Age*I and *Eco*RI. This allows one to monitor the cutting efficiency of the vector by observing the release of the 1.9 kb fragment from the vector after digesting it with *Age*I and *Eco*RI. ORF of TetR was introduced into the downstream of PAC gene by fusing it with a F2A sequence to link PAC ORF. The TetR ORF has the same reading frame as PAC; as a result, this fusion protein will be post-translationally cleaved by F2A protease into TetR and PAC functional proteins.
TetO (a modified tetracycline repressor binding sequence); TetR (tetracycline repressor); PAC (Puromycin acetyltranferase).

Figure2

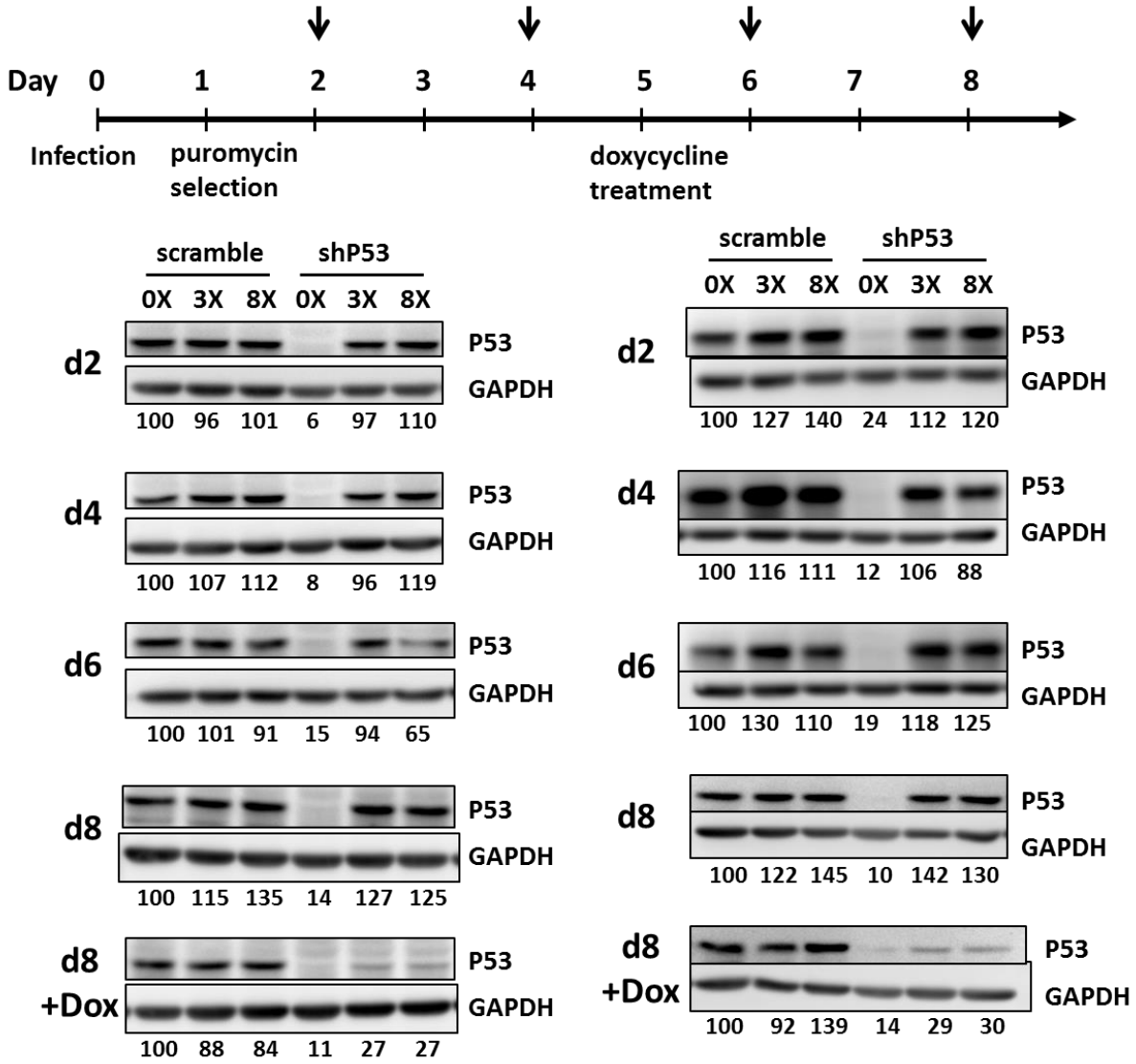


Figure 2. Tightness and responsibility of Tet shRNA inducible system. The results were obtained from two independent experiments. The quantitative P53 expression level normalized to GAPDH was shown under each of the Western blot. Western blotting lysate preparation. Cell was washed with cold PBS, scraped, 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.

VI. Appendix

Materials

Polybrene (Hexadimethrine bromide; Sigma H9268) or Protamine Sulfate (Sigma #4026)
Puromycin Dihydrochloride (Sigma #P8833)
Doxycycline (?)
Cellytic M Cell Lysis Reagent (Sigma #C2978)
cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche #04693132001)

Links

Protocol for shRNA construction-II: annealing method link (http://rnai.genmed.sinica.edu.tw/file/protocol/11_shRNA%20construction_V3.pdf#page=3)

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)