Version 1 (04/11/11)

Instructions for establishment of inducible cell line through lentivirus vectors

Introduction

A lentivirus based transfer vector system is capable of delivering gene into host cells of dividing or non-dividing by integrating the transgene into host genome. In addition, complete integration of the whole viral genome including the expression cassette of interest into the chromosome ensures that both the transgene and selection marker can be co-expressed in every transduced cell. On the basis of this, stable pooled mixture cells can be directly applied to further experiments. Thus, this system provides a rapid approach to establish stably expressed cells.

The lentiviral-based inducible vector developed by the RNAi Core is a two-component system described as follows:

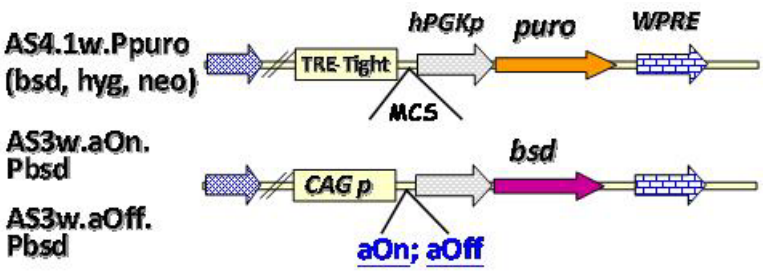
(a) The cDNA/ORF lentiviral transfer vectors based on tet-inducible system:

The tet-inducible vectors were modified from pLKO.1 lentiviral transfer vectors. It contains human PGK (pPGK) expression cassette directing expression of selection markers viz. puromycin (puro), blasticidin (bsd), hygromycin (hyg) or neomycin (neo/G418), respectively. Further these vectors are coupled with the new version of the TRE-tight promoter followed by the multiple cloning sites (mcs) for insertion of the gene/sequence of interest. These vectors can be directly used to prepare VSV-G pseudotyped lentivirus by following the standard protocol. Resulting virus(es) in combination with advanvced On (aOn) or advanced Off (aOff) lentivirus (see item ii below) provides a powerful tool for successful establishment of an inducible system.

(b) The aOn and aOff-expressing lentiviral vectors:

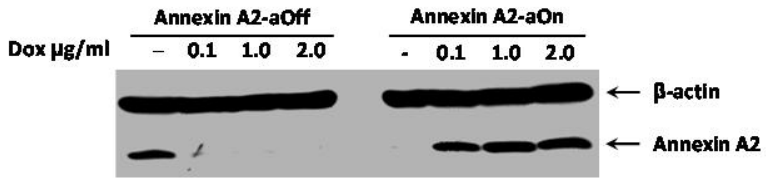
The aOn activates transcriptional activation of the TRE in the presence of doxycycline, an inducer of tet operator. The aOff, on the other hand, inactivates its activation in the presence of doxycycline. The expression cassette was also engineered on lentiviral transfer vector with blasticidin (bsd) selection marker. Thus aOn- or aOff- expressing stable cell line could be established easily by transducing cells with this lentivirus followed by selection against blasticidin.

Their maps are as follows:



To test the responsibility and tightness of these vectors, HEK293 cells were first transduced with

AS3w.aOn.Pbsd or AS3w.aOff.Pbsd lentiviruses followed by selecting with blasticidin for 10 days. Resistant cells were then transduced with lentivirus vector expressing Annexin A2 (on pAS4.1.Ppuro vector) followed by selecting with puromycin for three days. As shown in the following figure,



Annexin A2 was not detectable under non-induction conditions as demonstrated by adding doxycyclin in aOff system or without adding doxycyclin in aOn system. On the other hand, the amounts of Annexin A2 gave rise to a promising level after induction. Together, these data indicate that the lentiviral-based inducible system is suitable for rapid establishment of stable cell line in terms of responsibility and tightness of the inducible system.

Reagents available in the Core list as follows

(a) Lentiviral-based aOn or aOff expressing virus
C6-4-5: pAS3w.aOn.Pbsd lentivirus
C6-4-6: pAS3w.aOff.Pbsd lentivirus

(b) Tetracycline Inducible Transfer Vectors
C6-8-16: pAS4w.1.Ppuro
C6-8-17: pAS4w.1.Pbsd
C6-8-18: pAS4w.1.Phyg
C6-8-19: pAS4w.1.Pneo

Optimization of lentiviral infection

Parameters for lentiviral infections should be optimized for each cell line, such as cell seeding density, amount of lentivirus, and antibiotics concentration. The general guide lines for establishing tet-inducible cells by using these reagents are described as follows:

I. Materials
Human or mouse cell line and appropriate growth media
pAS3w.aOn.Pbsd or pAS3w.aOff.Pbsd lentivirus
tet-inducible lentivirus vectors.
6-cm tissue culture plates
12-well tissue culture plates
Polybrene (Hexadimethrine bromide; Sigma #H9268) or Protamine sulfate (MP Biomedicals #194729)
Blasticidin

Puromycin Dihydrochloride (Sigma #P8833)
Neomycin(G418)
Hygromycin (hyg)
Doxycycline (Dox)

II. Procedure

1. Seed cells at appropriate density in 6 cm plates with 5 mL media.
   (a) Adherent cells: seed 1 day prior to infection. Incubate overnight (37 °C, 5% CO2).
   (b) Suspension cells: seed at the day of infection in media containing polybrene.
2. Add lentivirus vector to cells:
   (a) Adherent cells:

   Remove growth media and add fresh media containing polybrene. Alternatively, remove a portion of the growth media and supplement with media containing polybrene. Adjust volumes and polybrene concentration to achieve the correct final polybrene concentration as below table (save one well without adding virus as negative and antibiotic control):

Reagent	Per 6 cm plate
Media containing polybrene*	to 5 mL
Final polybrene concentration	8 μg/mL
Virus: M.O.I = 2 -3	0.2 - 0.4 mL

   Note: Protamine sulfate can be substituted if polybrene is toxic to the cells.

   (b) Suspension cells:
   Add lentivirus vector (AS3w.aOn.Pbsd or AS3w.aOff.Pbsd) to the cells.

   Note: If available virus can not reach MOI=3, add the virus as much as you can; alternatively, seed the cells onto 6-, 12- or 24-well plate for transduction. The general principle is to select antibiotic resistant cells from the transduced cells. Therefore, the MOI used is not absolutely important for this purpose.
3. Spin Infection Option: For 12-well or 24-well plate transduction, spin cells at 2250 rpm (~1100Xg) for 30 minutes at 37 °C. Centrifugation can improve viral infection and decreases the length of exposure of cells to polybrene and virus.

   Note: Centrifugation is not recommended for 6-well plates or larger, as cells may not be fully covered with media during the spin
4. Incubate cells for overnight (37°C, 5% CO_2).
5. Change media 24 hours post-infection. Remove media and replace with 5 mL fresh growth media containing blasticidin (normally used at a concentration of 10 μg/ml) to select for stable transfectants.
   Note: Blasticidin concentration should be optimized for each cell line; Typically, concentrations between 2 and 10 μg/ml Blasticidin are sufficient for selection in mammalian cells.

6. Refresh medium containing blasticidin every 3-4 days.
   Note: Antibiotics work best when cells are actively dividing. If the cells become too dense, the antibiotic efficiency will decrease.
7. Observe the cells until the cells in control well are died. Cells may form into colonies with an additional week or more depending on the cell line and transduction/selection efficiency.
8. Pool the resistant cells to a 10-cm cell culture dish and maintain on selection medium for an additional 7 days. This pooled culture will be expanded for subsequent tet-inducible lentiviral infection.
9. Repeat the infection protocol by using the On- or Off-expressing stable cells generated and tet-inducible lentivirus vectors.
10. Change media 24 hours post-infection. Refresh growth media containing antibiotics (blasticidin, puromycin, hygromycin or neomycin) to select for stable transfectants.
11. Incubate cells at 37°C, 5% CO_2 and replace growth media containing antibiotic as needed (every 3-4 days). Selection periods are highly dependent on the antibiotics used. Puromycin selection requires at least 48 hours.
12. After selection, split the cells to 12-well plate. Add doxycycline at different concentration (e.g. 0.5, 1 and 2 ug/mL) to determine the optimal concentration of the dox.
13. Assay the tightness and responsibility of the stable cells established.