**Establishment of a cell line with inducible expression of H7N9 PA**

Preparation of pLV-EF1a-H7\_TetON3G-blast lentiviral expression construct

- subcloning TetON3G to pLV-EF1a-blast

- 5’ cloning site: BamHI

- 3’ cloning site: EcoRI

BamHI and EcoRI digestion of pcDNA3.1-TetON3G and pLV-EF1a-blast

|  |  |
| --- | --- |
| DNA | 1 ug |
| CutSmart buffer | 5 uL |
| BamHI-HF | 1 uL |
| EcoRI-HF | 1uL |
| ddH2O | Top up to 50 uL |

- incubate at 37ºC overnight

- ligation of TetON3G and pLV-EF1a-hygro and transformation to Stbl3 competent cells

- midiprep of plasmid DNA

Packaging of lentivirus

- seed 293FT on a 10cm dish

- one day after seeding, the confluence reaches 50% for transfection

- transfection of 3ug of TetON3G lentiviral expression plasmid and 9uL of ViralPower lentiviral packaging mix

- harvest the supernatant 48h post transfection, filter with 0.45um filter and add 2mL of PEG-it and incubate at 4ºC overnight

- on the next day, centrifuge the virus-containing supernatant at 3000g for 30 mins at 4ºC

- resuspend the virus-containing pellet with 100uL of PBS

Transduction of TetON3G lentivirus to MDCK-2P

- add 20uL of lentivirus to MDCK-PB2 on a 12-well plate

- change the medium 24h after transduction

- 48h post transduction, the cells were trypsinized and seeded on a 10cm dish together with a control plate with non-transduced cells

- on the next day, select the transduced cells with blasticidin at 7 ug/mL

- select the cells for ~5-7 days until all the cells on the control plate die

Cellular RNA extraction

- seed cells on a 6-well plate

- for each well, use 1 mL of RNAiso plus to lyse the cells

- pipette up and down until the cells are homogenized

- add 200uL of chloroform and incubate at room temperature for 2 mins

- centrifuge at 20,000g for 15 mins at 4ºC

- extract the liquid phase supernatant and transfer to 500uL of isopropanol with 1.5uL of Glycoblue

- incubate at room temperature for 10 mins

- centrifuge at 20,000g for 15 mins at 4ºC

- discard the supernatant and resuspend the pellet in 1mL of 75% ethanol

- centrifuge at 20,000g for 5 mins at 4ºC

- discard the supernatant and resuspend the pellet in 60uL of TE buffer

RT-PCR of cellular RNA

- template: cellular RNA

- primer: RT primer mix

- enzyme: PrimeScript RT reaction kit

- RT-PCR condition:

gDNA removal

|  |  |
| --- | --- |
| RNA | 1 ug |
| 5x gDNA eraser buffer | 2 uL |
| gDNA eraser | 1 uL |
| RNase-free water | Top up to 10 uL |
| Total | 10 uL |

- 42ºC for 2 mins

RT-PCR

|  |  |
| --- | --- |
| Reaction mixture from gDNA removal | 10 uL |
| 5x PrimeScript buffer 2 | 4 uL |
| RT primer mix | 1 uL |
| PrimeScript RT Enzyme Mix I | 1 uL |
| ddH2O | 4 uL |
| Total | 20 uL |

qPCR quantification of TetON3G expression

- template: cellular cDNA

- targets: PB1, PB2, TetON3G, ActB

- PB2 forward primer: cctgacatgacccccag

- PB2 reverse primer: gtcagcttttctgttccctg

- PB1 forward primer: aggcaagctgaaaaggagg

- PB1 reverse primer: gtgaaggagagctctgtatc

- TetON3G forward primer: accgctgtgctctcctctc

- TetON3G reverse primer: acttgatgctcctgttcctcc

- ActB forward primer: gatgagattggcatggcttt

- ActB reverse primer: caccttcaccgttccagttt

- 384-well PCR reaction plate

|  |  |
| --- | --- |
| cDNA (10-fold diluted) | 1 uL |
| 2x SYBR Premix Ex Taq II | 5 uL |
| RNase-free water | 2.8 uL |
| Forward primer (2.5uM) | 0.5 uL |
| Reverse primer (2.5uM) | 0.5 uL |
| ROX reference dye II | 0.2 uL |
| Total | 10 uL |

Applied Biosystems ViiA 7 Real-Time PCR System conditions

|  |  |  |
| --- | --- | --- |
| 95ºC | 30 secs | 1 cycle |
| 95ºC | 5 secs | 40 cycles |
| 60ºC | 30 secs |

Preparation of pLV-TRE3G-H7\_PA-neo lentiviral expression construct

- substituting the EF1a promoter of pLV-EF1a-neo to TRE3G by subcloning

- 5’ cloning site: ClaI

- 3’ cloning site: AgeI

ClaI and AgeI digestion of pcDNA3.1-TRE3G and pLV-EF1a-neo

|  |  |
| --- | --- |
| DNA | 1 ug |
| CutSmart buffer | 5 uL |
| ClaI | 1 uL |
| AgeI-HF | 1uL |
| ddH2O | Top up to 50 uL |

- incubate at 37ºC overnight

- ligation of TRE3G and pLV-EF1a-neo and transformation to Stbl3 competent cells

- midiprep of plasmid DNA

- PCR amplification of H7\_PA

- 5’ cloning site: AgeI

- 3’ cloning site: NotI

- forward primer: tagcaaccggtatggaggatttcgttagacaatgcttcaatccaatgatcgtcg

- reverse primer: tagcagcggccgctcacctaagagcgtgagtaaggaaggagttgaaccaag

- template: pHW2000-H7\_PA

- enzyme: KAPA HiFi HotStart PCR Kit

PCR reaction:

|  |  |
| --- | --- |
| Template | 0.5 uL |
| 5x KAPA HiFi buffer | 5 uL |
| KAPA dNTP Mix (10mM ) | 0.75 uL |
| Forward primer (10uM) | 0.5 uL |
| Reverse primer (10uM) | 0.5 uL |
| KAPA HiFi Hotstart DNA polymerase (1U/uL) | 0.5 uL |
| ddH2O | 17.25 uL |
| Total | 25 uL |

- PCR condition:

|  |  |  |  |
| --- | --- | --- | --- |
| Initial denaturation | 95ºC | 3 mins | 1 cycle |
| Denaturation | 98ºC | 20 secs | 25 cycles |
| Annealing | 60ºC | 15 secs |
| Extension | 72ºC | 2 mins and 15 secs |
| Final extension | 72ºC | 3 mins | 1 cycle |

- PCR product is gel cleaned for restriction enzyme digestion

- restriction enzyme digestion condition

|  |  |
| --- | --- |
| DNA | 1 ug |
| CutSmart buffer | 5 uL |
| AgeI-HF | 1 uL |
| NotI-HF | 1uL |
| ddH2O | Top up to 50 uL |

- incubate at 37ºC overnight

- ligation of PCR product and pLV-TRE3G-neo and transformation to Stbl3 competent cells

- midiprep of plasmid DNA

Packaging of lentivirus

- seed 293FT on a 10cm dish

- one day after seeding, the confluence reaches 50% for transfection

- transfection of 3ug of PB1 lentiviral expression plasmid and 9uL of ViralPower lentiviral packaging mix

- harvest the supernatant 48h post transfection, filter with 0.45um filter and add 2mL of PEG-it and incubate at 4ºC overnight

- on the next day, centrifuge the virus-containing supernatant at 3000g for 30 mins at 4ºC

- resuspend the virus-containing pellet with 100uL of PBS

Transduction of PA lentivirus to MDCK-2P-TetON3G

- add 20uL of lentivirus to MDCK-2P-TetON3G 12-well plate

- change the medium 24h after transduction

- 48h post transduction, the cells were trypsinized and seeded on a 10cm dish together with a control plate with non-transduced cells

- on the next day, select the transduced cells with geneticin at 800 ug/mL

- select the cells for ~7 days until all the cells on the control plate dies

Western blot analysis of inducible PA expression

- MDCK-3P cells were seeded on a 6 well plate

- add 1ug/mL of doxycycline to the cells

- cells were harvested at 6h, 24h and 48h

Harvest of cellular protein

- remove the medium and add 1mL of PBS

- remove the cells with a cell scraper and centrifuge at 2000g for 3 mins to collect the cell pellet

- lyse the cell pellet with 150uL of RIPA buffer for 30 mins on ice

- centrifuge at 20,000g for 10 mins

- collect the supernatant and perform protein quantification with Bradford using a spectrophotometer

- normalize the protein concentration to 1 ug/uL

- add 5x protein sample buffer (PSB) to the samples and boil for 10 mins

SDS-PAGE and Western blot

- prepare 10% resolving gel

- add 70% ethanol to overlay the resolving gel until it solidifies

- remove the ethanol and overlay the resolving gel with stacking gel

- assemble the gel and fill the gel tank with SDS running buffer

- for each gel, apply 20mA current for an hour

- semi-dry transfer with Power Blotting Station

- place the gel on top of the PVDF membrane activated by methanol

- sandwich the gel and membrane with absorbent sheets soaked with transfer buffer

- select the 10 min high molecular weight transfer program

- block the membrane with 5% skim milk for one hr

- binding of primary antibody (PA 1:1000; ActB 1:5000) at 4ºC overnight

- wash with TBST for 20 mins, 3 times

- binding of secondary antibody (HRP-conjugated sheep anti-mouse antibody 1:5000) at room temperature for 30 mins

- wash with TBST for 20 mins, 3 times

- develop chemiluminescence with WesternBright ECL system

- detect the signal with Sapphire Biomolecule Imager