Reprogramming of MEF into induced Pluripotent Stem (iPS) cells

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Materials:

1. uninducible system(OSKMLN system): pLenti-EF1a-cDNA-IRES-eGFP

   pLenti-EF1a-Oct4-IRES-eGFP

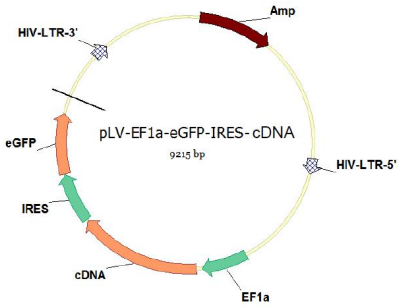
   pLenti-EF1a-Sox2-IRES-eGFP

   pLenti-EF1a-Klf4-IRES-eGFP

   pLenti-EF1a-cMyc-IRES-eGFP

   pLenti-EF1a-Lin28-IRES-eGFP

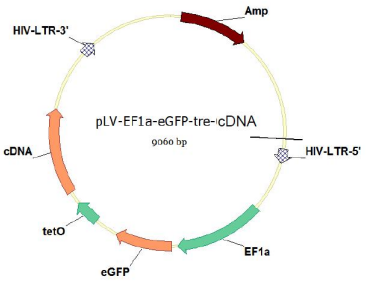
   pLenti-EF1a-Nanog-IRES-eGFP

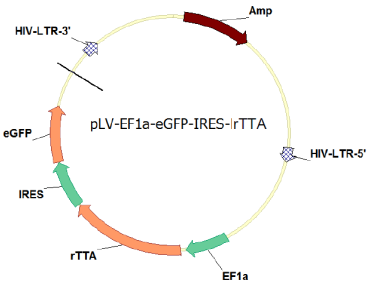


2. inducible system(OSKMR system): pLenti-EF1a-eGFP-tetO-cDNA
   and pLenti-EF1a-rTTA-IRES-eGFP

   pLenti-EF1a-eGFP-tetO-Oct4
   pLenti-EF1a-eGFP-tetO-Sox2
   pLenti-EF1a-eGFP-tetO-Klf4
   pLenti-EF1a-eGFP-tetO-cMyc

   pLenti-EF1a-rTTA-IRES-eGFP





3. MEF medium
   a. 10% FBS
   b. 88% DMEM (high-glucose)
   c. 1% P/S (5000 mg/ml penicillin and 5000 mg/ml streptomycin)
   d. 1% L-glutamine (200 mM)

4. 293T medium
   a. 10% FBS
   b. 88% DMEM (high-glucose)
   c. 1% P/S (5000 mg/ml penicillin and 5000 mg/ml streptomycin)
   d. 1% L-glutamine (200 mM)

5. mouse iPS medium
   a. 15% ES-FBS
   b. 82% DMEM (high-glucose)
   c. 1% P/S (5000 mg/ml penicillin & 5000 mg/ml streptomycin)
   d. 1% L-glutamine (200 mM)
   e. 1% NEAA(100 × MEM nonessential amino acids (Invitrogen, cat. no. 11140-050)
   f. 2-mercaptoethanol (2-ME; Sigma, cat. no. M-6250), to final 10^{-4} M
   g. mouse LIF, to final 10^{3} U/ml

6. mouse embryonic fibroblast(MEF); ≤P3

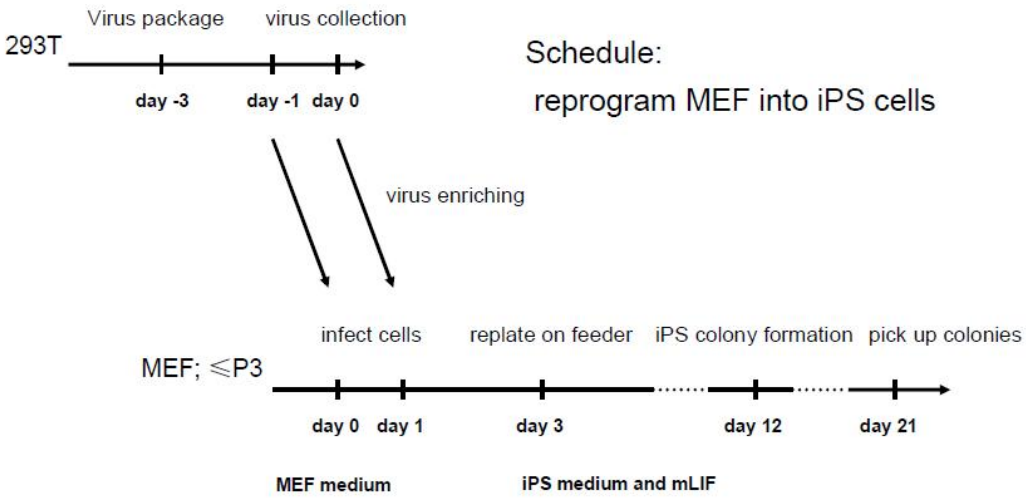
7. Dox(doxycycline); 8 mg/ml stock in H_2O, keep in dark, store at 4℃

8. polybrene; 8 mg/ml, store at 4℃

9. Mitomycin C; 1.2 mg/ml stock, store at -20℃

10. ultracentrifuge tube(Beckman, cat.no. 343060) and SW40 rotor

Methods:



Day -4

It's strongly suggested that beginning this step at 22:00.

replate 293T: passage (3:1) 2x 100mm fully covered dish of 293T to 6x 100mm dish.

Day -3

(15h later)PEI transfection: add 8ug(cDNA):3ug(psPAX2):1.5ug(pMD2G) into 1ml opti-MEM; add 50ul 1x PEI reagent then pipet mix immediately and carefully, do not pipet out bubbles. let still at

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room temperature for 20min then transfer all into the 100mm dish which replated the previous day, agitate-mix softly(note 1).

Day -1

Collect and pellet lentivirus: (48h later)using syringe to collect viral liquid and pass it through 0.45um filter into ultracentrifuge tube(Beckman, cat.no. 343060) then fill up with 3ml mineral oil; resupply with 10ml 293T medium. Load ultracentrifuge tubes into corresponding hanging tube, accurately balance them with mineral oil(note 2). 50 000 x g, 1.5h, 4℃, with no brake. carefully discard all mineral oil, and then discard all supernatant. add 100ul DMEM to pellet and let it still over night at 4℃.

passage and seed 20 000 MEFs(≤P3) onto each 24 well-plate wells

Day 0

repeat Day -1 virus collection and ultracentrifuge.

Lentivirus infection: carefully pipet to resuspend viral pellet , mix viral liquid (uninducible system:OSKMLN or inducible system: OSKMR) to viral cocktail. add 400/40/4ul cocktail to corresponding 24well-plate well and add 1.4/1/1 ul 8mg/ml polybrene to corresponding well and make polybrene final concentration 8ug/ml. agitate mix, 37℃,5% CO_2.(note 3)

Day 1

refresh MEF medium and repeat Day 0 virus infection

thaw MEF and plate on 6 well-plate wells. these MEF will later act as feeder layer.

Day 2

refresh both feeder MEF medium and reprogramming MEF medium.

Day 3

Mitomycin C treatment of feeder layer: pipet away medium from feeder MEF, add 800ul MEF medium and 8ul 1.2mg/ml mitomycin C(to a final concentration 12ug/ml). Agitate mix well and incubate for 3h(5% CO_2, 37℃), then remove medium and PBS wash three times; resupply another 2ml fresh iPS medium.

Replate reprogrammed MEF on feeder: aspirate medium from reprogramming MEF. PBS washed and then add 100ul 0.25% trypsin with EDTA incubate at 37℃ for 5min, then add 1ml MEF medium to end digestion. Collect cell suspending medium and spin down at 300 x g, 4min. Discard all supernatant and resuspending in 1ml fresh iPS medium (2 x mLIF, if inducible system add with Dox to a final concentration of 4 ug/ml),transfer it to feeder(medium aspirated) and add another 1ml iPS medium. Agitate mix well. From now on, culture with iPS medium(5.5% CO2, 37℃).

Day 4-6

refresh medium everyday(iPS medium: supplied with 2 x mLIF, if inducible system add with Dox to a final concentration of 4 ug/ml). 5.5% CO2, 37℃

Day 7 and to the end

refresh medium everyday(iPS medium, if inducible system add with Dox to a final concentration of 4 ug/ml). 5.5% CO2, 37℃

From this day on, can observe significantly GFP expression (395nm)

Day 12(about)

Can see iPS colony formation

Day 21

Can pick up iPS colony and digest it, replate to fresh feeder, culture with iPS medium, 5.5% CO_2, 37℃

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Notes:

1. 293T cells do not adhere dishes tightly; it's very easy to pipet up from the dishes, so manipulate it with care.
2. The discrepancy of two opposite hanging tube should be less than 0.003g.
3. Viral titer dominates iPS efficiency.

Virus volume (μl) required = [(MEFs seeded for infection)/virus titer (U/ml)] x [(desired MOI)/1 ml] x 1000ul.

For example, if the number of cells in the well at the time of transduction is $1x\ 10^5$, the viral titer is 3×10^8 IFU/ml, and a desired MOI is 20, then the volume of virus required is:

$(1 \times 10^5$ cells)/$(3 \times 10^8$ U/ml) x [20/1 ml x 1000ul] = 6.6 μl virus required for 1 well of a 6-well plate.

Try these concentration gradient if you are not sure how much these viral titer is.