**Retrovirus production**

**Transfection**

Day1

Culture Phoenix cells to 50-60% of confluency in 10cm plate

**(Yamanaka used 0.05% trypsin/0.53mM EDTA to culture pMX cells)**

Put 20ug of plasmid DNA (Oct4, Sox2, Cmyc or Klf4, individually!!!) + 10ug of helper plasmid + 62ul of CaCl2 + water (total volume should be 500ul) in FACs tube (16ug of DNA/8ug helper also fine)

(Jaenisch used 10ug of DNA

Make bubbles while you add 500ul of 2xHBS drop wise. Make bubbles to another 5-10seconds after adding the 500ul x2HBS

Wait 5 min (the solution will look cloudy). Use a blank tube + water as comparison

Change phoenix media including 2.5ul chloroquine in 10ml of media.

Add the 1ml of the transfection solution to the plates very very gently
(Just drop it around the plate all the area with 1ml of pipette and then shake the plate several times back and forth until the solution spread evenly) Do not swirl.

Day2
To remove CQ, aspirate the transfection solution-containing media and then add 10ml of fresh media 8-12 hrs later

Before leaving, aspirate the media and then put 5ml (3-6ml, it depends on how much concentrated you need) of fresh media for infection

**Infection (2 infection needs to be 6hr apart)**

Day1

Culture primary MEF to -90% confluency in 10cm dish

Day2 or 3 (Depend on primary MEF confluences)

Plate 1.3x10^5 MEF on a gelatin-coated well of 6well plate

(Decreasing 30% might be better ?)

(Jaenisch plated 4x10^5 cells/10cm plate to infection and one time infection)

(Yamanaka used 0.05% trypsin/0.53mM EDTA and plated on feeder cells which is mitomycin C-inactivated SNL cell and one time infection)

Day 4

Collect all the media from phoenix cell plate (48hrs after transfection) by using 10ml syringe, filtering it through 0.45um pore size filter and then transfer into 50ml tube

(Combine all the media with equal volume)

Add polybrene (1000x) in virus containing media and gently mix it.

Dilute with fresh media (1:1 but if you need more concentrated virus containing media, you can make 4 (virus):1(media)

Aspirate the media from MEF plate and then add 2ml of combined virus containing media

Spin 3min 1000rpm

Incubate overnight

**(At this stage, cells can be frozen based on Jaenisch's protocol)**

**Sorting**

Day 5

To remove polybrene change media (iPS media)

Day 7

Change media (iPS media)

Yamanaka used ES media and then started selection with the ES media including 0.3mg/ml of G418

Day 9-

(You have to prepare feeder cell plate to put sorted cell. The plate depend on your plan)

Sorting GFP+ cells by FACs

(You can put the sorted cell into the plate directly. But you should change iPS media into the plate 2hrs ago)

Ex) 1 cell/well of 96well plate

5000 cell/well of 24well plate

Change media every day until the colonies become big enough to be picked up or quantify. Colonies should first become visible approximately a week after the retroviral infection. They should become large enough to be picked up around day 20.

**George Daley splitted all infected cells into plate with iMEF.**

**Picking up iPS colonies**

(You must change media in irradiated MEF feeder cell plate and iPS plate with iPS media, transfer before 2hrs)

Put the microscope into culture hood after clean using 70% EtOH

Take the time around 20-30 min with UV

Put the plate on the microscope

Remove the media from the plate and add PBS (<1ml, may be less than 0.5ml) after wash one time with 1xDPBS

Pick the colonies from the plate using pipet set at -10ul and transfer the colonies into 96well plate including 50ul of 0.25%trypsin/1mM EDTA (Pick up as many good colonies as you can within 20 min)

**(Yamanaka picked colony using pipetman set at 2ul and then transfer it into 96well plate including 20ul of same trypsin)**

Incubate 10-15min or 7min in trypsin at 37°C and then pipette up and down several times

Spread the cells onto irradiated MEF feeder cell plate which is 24 well plates and then add 440ul of iPS media to each well (You can try 96 well plate to make cell line)

**For Mouse iPS cell (15 % FBS)**

| Name of component | Volume | Cat # |
|---|---|---|
| KO DMEM | 500 ml | |
| FBS | 90 ml | 16141-079<br>GIBCO<br>(Lot 522207) |
| NEAA (100x) | 6 ml | 11140<br>GIBCO |
| Glutamine | 6 ml | |
| P&S | 6 ml | |
| β-ME | 3.7 ul | |
| LIF | 60 ul | ESG1107<br>CHEMICON |

Materials: 0.25% Trypsin-EDTA 1x (GIBCO 25200)

## Transfering Mouse iPS (Trypsin method)

(You must change media in irradiated MEF feeder cell plate and iPS cell plate with iPS media 2hrs ago)

Remove the media on the plate and then add Trypsin (1ml/10cm plate) after wash with DPBS

7-15mins in a 37°C incubator

**(In case of WT iPS cell, 7-10 min is totally enough. But others such as p53KO iPS cells are needed more time)**

Put the iPS media and resuspend the cells gently

Split the contents at ratio of 1:4 or 1:5 onto plates with irradiated MEF feeder cells

**(If the cells are not confluent, you can split them at 1:3 – 1:1)**

## Transfering Mouse iPS (Hand picking method)

(You must change media in irradiated MEF feeder cell plate and iPS cell plate with iPS media 2hrs ago)

**(I recommend this method when the most of iPS cells are not showing good quality of morphology)**

Put the microscope into culture hood after clean using 70% EtOH

Take the time around 20-30 min with UV

Put the plate on the microscope

Transfer 100ul of 0.25%trypsin/1mM EDTA into 1.5ml tube and incubate 10 min

Remove the media from the plate and add DPBS (<1ml, may be less than 0.5ml on a well of 6 well plate) after wash one time with 1xDPBS

Pick the all colonies that showing good morphology from the plate using 2ul tip connected with 200p and 1ml tip and then transfer the colonies into 1.5ml tube including 100ul of 0.25%trypsin/1mM EDTA

**(Pick up as many good colonies as you can within 15 min)**

Swirl the plate to gather the colonies on the middle area.

Take the colonies using 200p pipette set at 100ul

Incubate the tube 5-10 min

Pipette up and down gently to resuspend the cells

Spread the cells onto irradiated MEF feeder cell plate

**(Ratio is depend on how many colonies did you pick up)**

## Freezing Mouse iPS

(Change media 2 hour before freezing the cells)

- Aspirate off media and then wash iPS using PBS
- Aspirate off PBS and then add 0.25% Trypsin (1ml/10cm plate)
- 10mins in a 37°C incubator
- Add iPS media and suspend the cells gently by pipetting
- Spin iPS at 800rpm 5min

- Discard supernatant and put freezing medium (10% of DMSO, 10% of FBS in iPS media) to the concentration at $2x10^6$ cells per ml
- Aliquot iPS at 0.5ml per vial
- Put vials in cell-freezing container and keep it at -80°C overnight
- Transfer vials to liquid nitrogen tank

## Thawing Mouse iPS

(You must change media in irradiated MEF feeder cell plate with iPS media, transfer before 30 mins)

- Thaw the vial into 37°C incubator quickly
- Put 2-3 drops of iPS media gently and slowly and then put more iPS media
- Transfer it into 15ml conical tube and then make 10ml
- Spin 800rpm 3-5 min
- Aspirate off the supernatant and then put iPS media and resuspend iPS gently
- Spread onto irradiated MEF feeder cell plate

**For irradiated MEF feeder cells**

| Name of component | Volume | Cat # |
|---|---|---|
| DMEM (high glucose) | 500 ml | 12430 GIBCO |
| BS | 25 ml | |
| Antibiotics | 5 ml | |

When you make feeder, plate cells with this media to the concentration at $1x10^6$ cells per 10cm culture dish or $1.5x10^5$ cells per well of 6 well plate