Title	Reprogramming human fibroblasts using an episomal vector system
Date Submitted	May 3rd, 2012
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Adapted from -	A more efficient method to generate integration-free human iPS cells. Okita et al. Nat Methods. 2011. 8:409-12.
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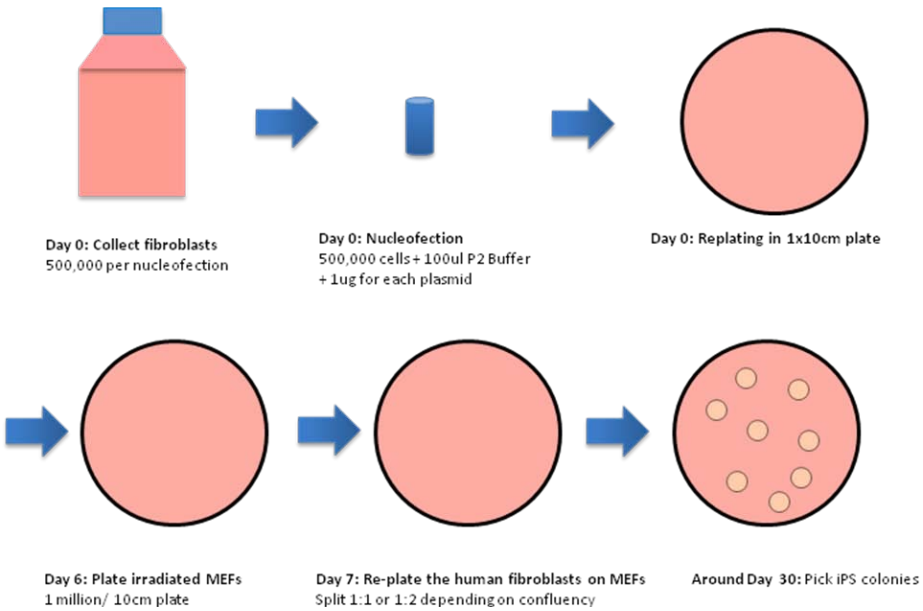
- Introduction: In 2006, Dr. Yamanaka showed that somatic cells could be reprogrammed into pluripotent stem cells by introducing 4 factors: Oct4, Sox2, Klf4 and c-Myc. Most of the early reports on somatic cell reprogramming used retroviruses or lentiviruses to achieve a sustained expression of these genes. However, the iPS lines generated through these virus systems have random integration of the provirus into the genome and therefore are not transgene free. Using non-integrative episomal vectors to introduce the reprogramming factors allows the derivation of transgene-free iPS lines.

- Flow Chart:



- Protocol:

- Media Preparation

  -Media component details

bFGF	100ng/ul

- Thawing Fibroblasts (4 to 5 fibroblast samples at one time)
  - Set up 4 to 5 x 15 ml conical tubes (one for each sample).
  - Partially thaw the frozen vial of fibroblast cells at 37ºC, until there is a small piece of ice remaining. Spray the vial with 70% ethanol to sterilize.
  - Take 1 ml of pre-warmed fibroblast medium, slowly add to the vial and transfer the liquid content with cells into the 15 ml tube. Slowly add 9ml dropwise.
  - Spin at 1200 RPM for 4 min.
  - Aspirate the medium from the spun down tube, and gently resuspend the pellet with 7-10 ml of fibroblast medium. Transfer the cells onto one T25 flask (or as recommended on the vial).
  - Change the medium the following day.
  - Before collecting the cells for nucleofection, we highly recommend to test them for mycoplasma. Several kits are commercially available. We routinely use the Lonza kit (Myco Alert).
  - When cells are almost confluent and tested negative for mycoplasma, they can be collected.

- Trypsinizing fibroblasts
  - Remove fibroblast medium
  - Wash once with PBS
  - Add 3-4ml of Trypsin/EDTA for each T25 flask.
  - Place in the incubator for 4 minutes
  - Detach the cells by tapping the flask
  - Add 4 ml of fibroblast medium to inhibit the trypsin
  - Collect all the cells and transfer into one 15ml tube
  - Spin at 1200 RPM for 4 min.
  - Aspirate the medium from the spun down tube, and gently resuspend the pellet with 5 ml of fibroblast medium without pen/strep.
  - Count cells

- Fibroblast nucleofection:

  - For each sample, transfer 500,000 human dermal fibroblast cells into an eppendorf tube
  - Spin down the eppendorf tube for five minutes using the table top centrifuge.

- Aspirate the supernatant
- Add 1ug of each plasmid (three plasmids:)
- Add 100ul of P2 Nucleofection Solution (82ul of solution +18ul of supplement)
- Rapidly transfer into the nucleofector cuvette.
- Nucleofect using the program NHDF.
- Quickly add 500ul of Fibroblast medium without pen/strep to the cuvette
- Collect the cells using a pipette
- Plate onto a 10cm dish and add Rock inhibitor (Y27632) at 10uM.
- The next day (day 1) change to fresh fibroblast medium with Pen/Strep.
- In the following days, change medium every other day.

➢ Plating MEFs, on day 6:
- Prepare 1x10cm plate for each sample.
- Add 0.1% gelatin on each plate to cover the surface (~4ml)
- Leave the plate at room temperature for 20-30 minutes
- Remove gelatin
- Plate 1million irradiated MEFs into each plate

➢ Replating fibroblasts on MEFs, on day 7:

- Remove Fibroblast medium from each plate.
- Wash once with PBS
- Add 4 ml of 0.25% trypsin/EDTA
- Place in the incubator for 4 minutes
- Add 4 ml of fibroblast medium to inhibit the trypsin
- Detach the cells by pipetting up and down. If necessary use a cell scraper to detach all the cells.
- Collect the cells and transfer into one 15ml tube
- Spin at 1200 RPM for 4 min.
- Aspirate the medium from the spun down tube, and gently resuspend the pellet in 10 ml of iPS medium.
- Remove medium from the MEFs plates
- Transfer the cells from each sample into one 10cm plate.

➢ Switch to hiPS medium

- The following day, the fibroblasts should be attached to the MEFs. Remove the fibroblast medium and add 10 ml of hiPS medium with FGF (10ng/ul).

- Change medium every other day the first week and then every day. iPS colonies will start appearing around week 3-4 after transduction. However, many partially reprogrammed colonies can be seen earlier.

➢ Picking colonies:

- Pick only colonies that are fully reprogrammed based on morphology or TRA-1-60 positive Live Stain. It is better to wait until the colonies are big to do the picking. The colonies can be plated on MEFs (1 well of a 6-well or 12-well plate) or directly on Matrigel with mTeSr1 (feeder-free culture system). Rock inhibitor (Y27632) at 10uM should be added to the medium to increase survival.
- Each colony will be one line.

❖ Materials:

Reagents and plastics:

Irradiated MEFs. GlobalStem cat# 6001G	1 million/10cm plate
0.1% gelatin: Millipore cat# ES-006-B	4ml/10cm plate
pCXLE-hOCT3/4-shp53-F	
pCXLE-hSK	
pCXLE-hUL	
Nucleofector™ Kits for Human Dermal Fibroblast (NHDF)	
T25 flask	1 per sample
10cm dish	2 per fibroblast sample
15 ml tubes	
50 ml tubes	
Pipets	

MEDIA 1: MEF Media (500 ml)

DMEM	450ml
FBS	50ml
Pen/Strep	5ml
Glutamine	5ml

MEDIA 2: Standard hiPS Media (500 ml)

DMEM/F12	400ml
KO-SR	100ml
Pen/Step	5ml
Glutamine	5ml
MEM-NEAA	5ml
2-Mercaptoethanol (X1000)	500ul

Add bFGF just before feeding the cells (10ng/ml)

- Troubleshooting:

  -You can test the nucleofection efficiency using an episomal vector containing GFP. In our hands, the efficiency is around 30-40%.
  -We usually see a lot of cell death following the nucleofection. The fibroblasts recover well after 5-6 additional days in culture. Then, we transfer them to MEFs plates.

- References:

  - A more efficient method to generate integration-free human iPS cells. 2011 Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. Nat Methods. 8:409-12.

- Acknowledgements:

- Comments